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Copper Curcuminoids Containing Anthracene Groups: Fluorescent Molecules with Cytotoxic Activity

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The coordination chemistry of the new curcuminoid ligand, 1,7-(di-9-anthracene-1,6-heptadiene-3,5-dione), abbreviated 9Accm has been studied, resulting in two new copper-9Accm compounds. Compound **1**, [Cu(phen)Cl(9Accm)], was synthesized by reacting 9Accm with [Cu(phen)Cl_2] in a 1:1 ratio (M:L) and compound **2**, [Cu(9Accm)$_2$], was prepared from Cu(OAc)$_2$ and 9Accm (1:2). UV−vis, electron paramagnetic resonance (EPR), and superconducting quantum interference device (SQUID) measurements were some of the techniques employed to portray these species; studies on single crystals of free 9Accm, [Cu(phen)Cl(9Accm)] and [Cu(9Accm)$_2$(py)] provided detailed structural information about compounds **1** and **2·py**, being the first two copper-curcuminoids crystallographically described. In addition the antitumor activity of the new compounds was studied and compared with free 9Accm for a number of human tumor cells. To provide more insight on the mode of action of these compounds under biological conditions, additional experiments were accomplished, including studies on the nature of their interactions with calf thymus DNA by UV−vis titration and Circular Dichroism. These experiments together with DNA-binding studies indicate electrostatic interactions between some of these species and the double helix, pointing out the weak nature of the interaction of the compounds with CT-DNA. The intrinsic fluorescence of the free ligand and both copper compounds provided valuable information over the cellular process and therefore, fluorescence microscopy studies were performed using a human osteosarcoma cell line. Studies in vitro using this technique suggest that the action of these molecules seems to occur outside the nuclei.

Introduction

In the search toward new metallic species with biological applications, copper compounds have proved to be excellent candidates.[1−3] In vitro tests have shown that compounds of this metal are capable of inhibiting proteasome in an irreversible manner. This interaction induces ubiquitin protein accumulation, leading to apoptosis in tumor cells. The overall process can be enhanced by certain organic groups, such as 8-hydroxyquinoline.[4] In addition, in vivo experiments have confirmed the cytotoxic properties of a number of copper compounds, stressing the intrinsic relation between the nature of their ligands and the final activity.[4,5] Regarding this matter, one of the most relevant examples is the 1,10-phenanthroline copper compound, [Cu^{II}(phen)$_2$Cl]Cl. This compound was the first Cu species described to show DNA-cleavage action.[6−8] Within the cell, [Cu^{II}(phen)$_2$Cl]Cl is reduced to [Cu^{I}(phen)$_2$Cl]. This species, subsequently, binds reversibly to DNA in a non-covalent fashion and later gets oxidized, thereby forming reactive species capable of cleaving single strands

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of DNA.[9,10] The copper concentration inside cells treated with $[Cu(phen)_2Cl]Cl$ is about 10-fold higher than the ones treated with standard Cu^{2+} salts, suggesting that the lipophilic phenanthroline moiety helps the copper to pass the cell membranes.[11] More recently, a related compound, copper bis-phenanthroline-coumarin, $[Cu(4\text{-Mecdoa})(phen)_2]$ (Mecdoa = coumarin), has been found as a potent antiproliferating agent, leading to cell apoptosis, or necrosis, with IC_{50} values just 10% smaller than that of cisplatin.[12] With these examples our goal is to highlight not only the potential use of copper compounds as anticancer agents, but also the implication of its ligands in its biological activity.

Following this idea, numerous aromatic, planar organic species are known with remarkable biomedical applications. Among them, *curcumin* is an exceptional drug. Well known since ancient times in traditional medicine, it has been only in the recent decades that it has triggered the interest of the scientific community, mainly because of its antioxidant and scavenger properties.[4,5] Originally, this substance is found in the rhizomes of the plant *Curcuma longa* from where it can be extracted. Despite that, several papers in the literature display methodologies for its achievement in high yields.[13] These procedures have endorsed the creation of a versatile family of related compounds, the so-called *curcuminoids*. Curcumin and curcuminoids are used in medical treatments as anti-inflammatory agents[14−16] and also in therapy against HIV, rheumatoid arthritis, multiple sclerosis, and Alzheimer disease.[17] In addition, some of them exhibit antispasmodic, antimicrobial, and antiparasitic properties as well.[18−20] Along with these advantages, these aromatic organic compounds are widely studied in the prevention and treatment of several cancers.[21,22]

Earlier in vitro studies have shown that the simultaneous administration of curcumin and copper improves the antioxidant and antitumoral properties of the free species.[23−25] Besides, in the presence of Cu^{II} salts, curcumin is capable of cleaving DNA, a consequence that has been associated with the formation of reactive oxygen species (from the phenolic groups of curcumin) not present with other metals, such as iron or nickel.[26,27] Therefore, there is a logical interest in the synergy of both, Cu^{2+} and curcumin or curcuminoid, and their biological effects. However, so far only a few examples of copper/curcumin and copper/curcuminoid compounds have been described.[23,28,29] Considering all the views exposed above, the present work presents the physical description of a novel curcuminoid, 9Accm (Figure 1); the synthesis and characterization of two new copper compounds containing this curcuminoid as a chelating ligand, that is, compounds **1**, [Cu(phen)-Cl(9Accm)], and **2**, $[Cu(9Accm)_2]$, and the biological studies of these species. Whereas extensive bibliographic data is available on the synthesis of curcumin and curcuminoids, only few of these species have been crystallographically portrayed.[30,31] Furthermore, previous to this work, just one reported structure of a compound was known, where a curcuminoid was attached to a ruthenium center.[32] Thus, we felt the need to provide detailed characterization on the structure of a novel curcuminoid, 9Accm, upon and after coordination, as well as introduce compounds **1** and **2** as the first copper/curcuminoid structures described so far. The structure of the free ligand was also determined for reference purposes. The characterization of compounds **1** and **2** provides information on the coordination of the new curcuminoid with copper, as well as its effect versus phenanthroline through comparison between compounds **1** and **2**. Finally, the anticancer activity of these systems will be shown together with studies on their interactions with DNA. So, the ligand 9Accm was designed with the aim of monitoring the final compounds in living cells using fluorescent microscopy. Our purpose in this paper is to present a comprehensive portrait of these new compounds, as well as providing more insight on their mode of action.

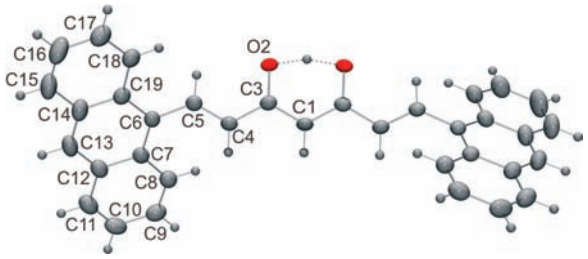

Figure 1. POV-Ray representation of 9Accm. Carbon and hydrogen atoms are in gray and oxygen in red colors.

Experimental Section

Starting materials were purchased from Aldrich, and all manipulations were performed using materials as received. $[Cu(phen)Cl_2]$ was synthesized as reported elsewhere.[33]

Preparation of 1,7-Di-9-anthracene-1,6-heptadiene-3,5-dione (9Accm). One gram of Hacac (10 mmol) and 0.5 g of B_2O_3

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(7.1 mmol) were dissolved in 10 mL of EtO_2CMe. The reaction mixture was heated at 40 °C for 30 min. Then, a solution of 4.1 g of 9-anthracenealdehyde (20 mmol) and 9.2 g of tributyl borate (4 mmol) in 20 mL of EtOAc was added. The mixture was stirred at 40 °C for 3 h. After cooling down, an excess of *n*-butylamine (0.5 mL, 50 mM) in EtO_2CMe (10 mL) was added dropwise. The final reaction was stirred at room temperature for 2 days. In the meantime, a dark red precipitate was formed. The colored solid was filtered and washed with MeOH to remove impurities. Yield: 51%. Crystals of 9Accm were achieved by layering a dichloromethane solution of the ligand with MeOH. IR (ν/cm^{-1}) 1625s, 1613s, 1442, 1131 ms, 995 m, 977, 958, 886 m, 845 m, 733vs, 541. Elemental analysis calculated for 9Accm·0.2MeOH ($C_{35.2}H_{24.8}O_{2.2}$): C, 87.54; H, 5.18 Found; C, 87.56; H, 5.71. ^{1}H-NMR (300 MHz DMSO-d_6, window from 12 to 0 ppm): δ 11.45 (s), 8.68 (m), 8.28 (m), 8.14 (m), 7.60 (m), 6.78(m).

Preparation of [Cu(phen)Cl(9Accm)] (1). 9Accm (25.0 mg, 0.05 mmol) was dissolved in dimethylformamide (DMF, 50 mL) and deprotonated with 2 mL of an aqueous solution of LiOH (3.75 mg, 0.16 mmol). Then, an equimolecular amount of [Cu(phen)Cl_2] (16.5 mg, 0.05 mmol) dissolved in DMF (15 mL) was added. The reaction mixture was stirred and heated at 80 °C for 3 h; later, a dark orange solid was obtained under reduced pressure. The powder was washed several times with water and diethyl ether and dried open to air. Yield: 40%. Crystals were obtained by layering a DMF solution of the solid with diethyl ether. IR (ν/cm^{-1}) 1623 m, 1541 m, 1505w, 1418w, 1170 m, 997, 872 m, 841 m, 736s, 720s, 454; Elemental analysis calculated for **1**·2H_2O ($C_{47}H_{35}ClCuN_2O_4$): C, 71.39; H, 4.46; N, 3.54. Found; C, 71.38; H, 4.36; N, 3.17. ESI-MS: *m*/*z*: 718 [M]$^+$, 749 [M + MeCN]$^+$.

Preparation of [Cu(9Accm)$_2$] (2). The ligand, 9Accm, (25 mg, 0.05 mmol) was dissolved in DMF (10 mL) and deprotonated with base (1,8-diazabicyclo[5.4.0]undec-7-ene 98% ; 15.6 μL, 0.11 mmol). Then, a solution of [$Cu_2(O_2CMe)_4(H_2O)_2$] (4.5 mg, 0.025 mmol) in 5 mL of DMF was added. A brown-orange complex was obtained by rotary evaporation. The dry solid was washed with water, methanol, and ether and dried in the air. Finally, an orange-brown powder was obtained. Yield: 55%. Crystals were obtained by layering a pyridine solution of the solid with diethyl ether (**2·py**). IR (ν/cm^{-1}) 1625 m, 1541s, 1517, 1440w, 1165, 885 m, 733vs, 701, 471; Elemental analysis calculated for **2**·H_2O ($C_{70}H_{48}CuO_5$) C, 81.42; H, 4.69. Found; C, 81.09; H, 4.65. ESI-MS (DMF/MeCN): *m*/*z*: 1078 [M+2MeCN]$^+$ and 1141 [M+2MeCN]$^+$.

X-ray Structure Analysis. Because of the crystal size and shape (thin orange laths in both cases), data for the free ligand 9Accm and compound **1** were collected from synchrotron radiation sources on a Bruker APEXII-CCD diffractometer, respectively on Daresbury SRS station 9.8, from a silicon 111 monochromator (T = 150 K, λ = 0.6911 Å) for 9Accm, and on Advanced Light Source beamline 11.3.1 at Lawrence Berkeley National Laboratory, from a silicon 111 monochromator (T = 150 K, λ = 0.7749 Å) for **1**. The structure of 9Accm was solved with SIR92,[34] while that of **1** solved using the SHELX-TL suite.[35] Refinement on F^2 and all further calculations for both compounds were also carried out using the SHELX-TL suite.[35] For 9Accm, all non-hydrogen atoms were refined anisotropically. All hydrogens were found in one difference Fourier map, and refined with a riding model placed geometrically on their carrier atom, except that on O2 that was refined freely with its thermal parameter 1.5 times that of O2. For **1**, all fully occupied non-hydrogen atoms were refinement anisotropically, as well as a 50% occupied DMF. Hydrogen atoms were placed geometrically where possible, which was not possible on the DMF methyl groups. It was not possible to find them in the different map either, therefore they were omitted from the refinement. Geometrical and displacement parameter restraints were used in modeling the disorder, leaving a few not-ideal anisotropic displacement parameter max:min ratios around 5:1. Data for compound **2** were collected on a small yellow crystal on a Bruker Kappa-Mach3/APEXII-CCD diffractometer equipped with a Mo-target rotating anode setup and an Incoatec-Helios-Mirror monochromator. Final cell constants were obtained from a least-squares fit of several thousand strong reflections. The structure was readily solved with the Patterson method and subsequent difference Fourier techniques. Refinement on F^2 was performed with the SHELXTL suite.[35] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed at calculated positions and isotropically refined as riding atoms.

Through the paper, the labels of **1** and **2** refer to [Cu(phen)-Cl(9Accm)] and [Cu(9Accm)$_2$], respectively, without having taken into account molecules of solvents (concentrations in the biological part were obtained using elemental analysis values). However, in the crystallographic section, **2** has been described as **2·py** because of the coordination of a pyridine molecule to the copper molecule.

Physical Measurements. C, H, and N analyses were performed with a Perkin-Elmer 2400 series II analyzer. Electrospray mass spectra (ESI-MS) in DMSO, DMF, or MeCN were recorded on a Thermo Finnigan AQA apparatus. Infrared spectra (4000−300 cm^{-1}) were recorded on a Perkin-Elmer Paragon 1000 FTIR spectrometer equipped with a Golden Gate ATR device, using the reflectance technique (resolution 4 cm^{-1}). NMR spectra were recorded with a 300-MHz Bruker DPX 300 spectrometer with a 5-mm multinucleus probe. X-band powder EPR spectra were obtained on a Bruker-EMX*plus* electron spin resonance spectrometer (Field calibrated with DPPH (g = 2.0036). Simulations of the spectra were done using MATLAB and Easyspin 3.0. Magnetic susceptibility measurements were carried out on polycrystalline samples with a MPMS5 Quantum Design magnetometer in the range 2−300 K under magnetic field of 1.0 T. Diamagnetic corrections were estimated from Pascal Tables. A spectrofluorimeter (Cary, eclipse) was employed to measure the fluorescence spectra in a variety of organic solvents. The fluorescence quantum yield Φ_f was obtained using rhodamine 101 in air-saturated ethanol, Φ_f = 0.9, as reference.[36] Partition coefficient measurements are shown in the Supporting Information.

DNA Solutions. To study the interaction with DNA, a stock solution of calf thymus DNA (CT-DNA) was used. The CT-DNA was dissolved in a 10 mM phosphate buffer with 5 mM NaCl (pH 7.2) and kept overnight at 4 °C. The final concentration of DNA per nucleotide was calculated through the maximum absorbance at 258 nm of a 10-fold dilution of a stock solution. The absorption coefficient of CT DNA was taken from literature[37] as 6600 cm^{-1} M^{-1} per nucleotide.

Circular Dichroism. Circular Dichroism (CD) experiments were performed to study the influence of different concentrations of complex on CT-DNA. Solutions were made with a stock solution of the copper compounds and the free ligand in DMSO and aliquots of CT-DNA solution. The concentration of CT-DNA was kept constant at 75 μM. The relative amount of DMSO was kept constant at 5% of the total volume, with the exception of [Cu(9Accm)$_2$] (**2**) were, because of its poor solubility, the relative amount of DMSO was increased until 10% to achieve a lower R value, where the R value is [DNA] per nucleotide/[compound]. The R values ranged from 1.2 to 10. Samples were incubated at 37 °C for 1 h. After measurement the samples were kept overnight at room temperature and measured again after 24 h. Data were collected on a JASCO J-815 CD spectrometer using 2.0 mm cuvettes. A baseline subtraction was used, with the baseline being a blank sample of 10 mM phosphate buffer, 5 mM NaCl (pH = 7.2). The spectra were measured from 350 to 220 nm in steps of 0.5 nm and a scanning

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speed of 200 nm per minute, and the sensitivity was set to 5 mdeg. The spectra were averaged over a total of 16 scans.

UV−vis Titration. UV−vis tritration studies were performed to study the influence of different concentrations of DNA on the absorbance of the copper compounds. Solutions were made with a stock concentration of the copper species in DMSO (2−5%) and aliquots of the CT-DNA solution. Concentrations between 14 and 40 μM of [Cu(phen)Cl(9Accm)] (**1**), 2% DMSO, and 72 μM of CT-DNA (fixed) were prepared and studied. On the other hand, compound **2** was kept at a fixed concentration, 7.7 μM, 5% DMSO, meanwhile the concentration of DNA in solution was varied to get different R values (R = [DNA per nucleotide]/[complex]). Samples were measured from 200 to 800 nm. Data were collected on a Varian Cary 50 UV−vis spectrometer with new Fiber Optic Dip Probe accessory using 1.00 cm cuvettes. A zero/baseline subtraction was used with the baseline being a blank sample of 10 mM phosphate buffer, 5 mM NaCl (pH 7.2).

DNA Gel Electrophoresis. DNA gel experiments were performed based on DNA studies as described.[38] Aliquots from stock solutions of both copper compounds and the free ligand in DMSO were taken and diluted with a 0.5 M phosphate buffer pH 7.2 to yield solutions with complex concentrations ranging from 10 μM to 200 μM. The relative amount of DMSO was kept as low as possible (less than 5% of the total volume in the case of [Cu(phen)Cl(9Accm)] (**1**) and up to 35% in the case of [Cu(9Accm)$_2$] (**2**)). Samples of ΦX174 DNA containing 0, 12.5, and 25% DMSO were used as a reference. The DNA solution (40 μM bp ΦX174 DNA (Invitrogen) 7 μM 40 μM base pairs) in 84 mM phosphate solution with 6.3 mM NaCl pH 7.2 was added and incubated at 37 °C for 1 h. Prior to its loading on a 0.8% agarose gel containing 2.54 μM ethidium bromide, 4 μL of loading buffer (bromophenol blue) was added. The gels were run at a constant voltage of 80 V for 90 min in TBE buffer containing 2.54 μM ethidium bromide. The gels were visualized under a UV transluminator with Bio Rad imaging software.

DNA Unwinding. pUC19 plasmid DNA (50 μM bp) was incubated with the copper compounds in varying concentrations (0−55 μM) in the dark at 37 °C for 24 h. The samples were placed on ice, and an equal amount of loading buffer (0.1% bromophenol blue. 0.1% xylene cyanol) was added to each sample. The samples were loaded onto a 1% agarose gel and examined by the electrophoretic mobility shift assay in a TBE buffer for 3 h at 70 V. The resultant gels were stained with 0.5 μg/mL ethidium bromide and destained to remove the excess of ethidium bromide. The DNA bands were visualized with a transilluminator and imaged with a BioRad ChemiDoc XRS apparatus.

Cytotoxicity. Cytotoxicity of the copper(II) compounds was studied using a colorimetric microculture assay (SRB assay) in seven well-characterized human tumor cell lines containing examples of breast (MCF7 and EVSA-T), colon (WIDR), ovarian (IGROV), melanoma (M19 MEL), renal (A498), and non-small cell lung cancer (H226). These studies were performed at TEVA-Pharmachemie, The Netherlands. Cell lines WIDR, M19, A498, IGROV, and H226 belong to the currently used anticancer screening panel of the National Cancer Institute, U.S.A.[39] The MCF7 cell line is estrogen (ER)+/progesterone (pgR)+ and the cell line EVSA-T is (ER)-/(pgR)-. All cell lines were maintained in a continuous logarithmic culture in RPMI 1640 medium with Hepes and phenol red. The medium was supplemented with 10% FCS, penicillin 100 units/mL and streptomycin 100 μg/mL. The cells were mildly trypsinized for passage and for use in the experiments. On the first day 150 μL of trypsinized tumor cells (1500−2000 cells/well) were plated in 96-wells flat-bottom microtiter plates. The plates were preincubated 48 h at 37 °C, 8.5% CO_2 to allow the cells to adhere. On day two, a 3-fold dilution sequence of 10 steps was made in full medium, starting with the 1 mg compound/200 μL stock solution (in DMSO). Every dilution was used in quadruplicate by adding 50 μL to a column of wells and kept undisturbed for 5 days. On the 7th day the incubation was finished by washing the plate twice with PBS. Cytotoxicity was estimated by the microculture sulforhodamineB (SRB) test.[40] The absorbance was read at 540 nm using an automatic microplate reader (labsystems Multiskan MS). Data were used for construction of concentration response curves and determination of the IC_{50} value by use of Deltasoft 3 software.

Fluorescence. In vitro fluorescence studies were performed to study the behavior of these copper compounds in U2OS cells (human osteosarcoma cells). Final solutions were made from stock solution of the copper compounds in DMSO and serum free medium. The relative amount of DMSO in the final solutions was kept constant at 0.7% of the total volume. Cells were incubated at 37 °C with 28 μM or 10 μM complex solutions for 30 min, 1 or 3 h. After the solution was removed, the cells were washed twice with PBS and 2.0 mL of complete medium was added. Since the copper compounds are inherently fluorescent, no dye was added.

Fluorescence Microscopy experiments were performed on an Axiovert 135 TV (Zeiss, Jena, Germany) inverted microscope equipped with a 100 W mercury arc lamp for fluorescence excitation and bright field illumination for phase contrast images. The filter set to detect complexes fluorescence consisted of an hq 480/40 nm band-pass excitation filter, an hq 535/50 band-pass emitter filter, and a Q505 long-pass beam splitter. The temperature of the culture medium was controlled between 36 and 37 °C by a BIOPTECHS (Butler, PA) objective heater and a heated ring surrounding the culture chamber. Digital images were taken with cooled CCD camera (Photometrix PLX, Tucson, AZ) using SCILL Image software (Multihouse, The Netherlands).

Results and Discussion

Synthesis and Spectroscopic Analysis. The new curcuminoid ligand, 9Accm, was prepared by the modified method reported in the literature.[13,41,42] Like in previous works, the first step involves the formation of a boron complex of 2,4-pentanedione under mild conditions. This prevents Knoevenagel reactions at the C-3 atom ensuring condensation specifically on the terminal methyl groups once the mixture of 9-anthracenecarboxaldehyde and tributyl borate is added. Then, *n*-butylamine gets incorporated as catalyst. However, here the boron complex was found unstable, probably because of steric impediments from the anthracene groups (four for each boron species), and decomposed without the use of dilute acids, giving as a result the designed curcuminoid. Thus, the stirring of the reaction for 2 days facilitated the formation of 9Accm in good yields which precipitated out of the solution. The final, red solid was washed with MeOH and single crystals were obtained by layering a dichloromethane solution of the ligand with MeOH. Between the two tautomeric forms of 9Accm:enol and ketone, the first one prevails, as shown in the solid-state by X-ray crystallography (see below). This was corroborated in solution by ^{1}H NMR, for which the spectrum in

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Table 1. Crystal Data and Structure Refinement for the Free Ligand 9Accm and Compounds **1** and **2·py**

	9Accm	**1**	**2·py**
formula	$C_{35}H_{24}O_2$	$C_{51.5}H_{41.75}ClCuN_3O_{3.62}$	$C_{75}H_{51}CuNO_4$
FW	476.54	859.62	1093.72
space group	$P22_12_1$	$P\bar{1}$	$P\bar{1}$
a/Å	5.1382(7)	14.8837(19)	9.135(4)
b/Å	13.6449(18)	21.785(3)	11.707(5)
c/Å	17.294(5)	27.969(4)	25.643(9)
α/deg	90	99.181(2)	86.746(8)
β/deg	90	103.243(2)	85.276(9)
γ/deg	90	90.031(2)	81.373(9)
V/Å^3	1212.5(4)	8708(2)	2699.4(19)
Z	2	8	2
T, K	150	150	100
λ Å	0.69110	0.77490	0.71073
ρ_{calc}, g·cm^{-3}	1.305	1.311	1.346
μ mm^{-1}	0.096	0.769	0.461
$R1[I > 2\sigma(I)]$	0.0446	0.0620	0.0428
$\omega R2$ (all data)	0.1184	0.2069	0.1077

DMSO-d_6 displays two singlets at 11.45 and 6.78 ppm, assignable to the intrahydrogen bond and the methine proton of the enol form, respectively. The unsaturated beta-diketone was found soluble or partially soluble in most of the apolar solvents, including ethers and hexanes, and finally reactions using copper salts were attained in DMF.

As described above, compounds **1** and **2** were synthesized by reacting [Cu(phen)Cl_2] and [$Cu_2(O_2CMe)_4(H_2O)_2$] in 1:1 and 1:2 ratios, respectively. Both reactions were achieved with an excess of base, and the final compounds were isolated using the rotary evaporation system to concentrate the solutions. Suitable single crystals for X-ray crystallography of both compounds **1** and **2** were obtained from layers of DMF/diethyl Ether and py/MeOH, respectively. IR spectra of 9Accm and compounds **1** and **2** are explained in more detail in the Supporting Information. The UV−vis spectra of 9Accm and compounds **1** and **2** in DMSO exhibit a high-energy band between 250 to 300 nm corresponding to the $\pi\rightarrow\pi^*$ transitions of 9Accm (Supporting Information, Figure S1 and S2). In addition, the 9Accm spectrum shows another two bands at approximately 350 and 420 nm related to $n\rightarrow\pi^*$ transitions, similar to other curcuminoids reported in the literature.[23,42,43] Compounds **1** and **2** display the latter band (420 nm), while the former is minimized in both cases (350 nm).

Crystal Structures. Two of the crystal structures, that is, those of the free ligand and compound **1**, required the use of a synchrotron source, meanwhile the structure of compound **2·py** was obtained using a standard diffractometer. General crystal data information of these three species is presented in Table 1.

9Accm crystallizes in the orthorhombic space group $P22_12_1$ with two molecules in the unit cell. A POVRAY view of the ligand is depicted in Figure 1. Selected bonds and angles are given in Supporting Information, Table S1. The crystallographic structure shows a diarylheptanoid system attached at both ends to anthracene groups. In the structure, the enol- and keto- forms are indistinguishable as the asymmetric unit contains only half a molecule, and therefore a unique distance C−O of 1.294(3) Å is found. However, the conjugated π system of the linear chain is observed with

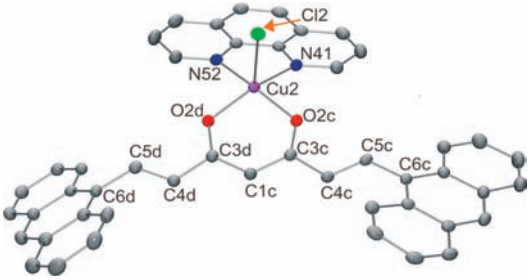

Figure 2. POV-Ray representation of compound **1**, [(phen)CuCl(9Accm)]. Ellipsoid probabilities are represented at 25%. Hydrogen and labels of the anthracene and phenanthroline groups have been removed for clarity. Carbon atoms are in gray, oxygen in red, nitrogen in blue, chloride in green, and copper in purple colors.

values that alternate between 1.33 and 1.45 Å. As expected, similar variations were observed in both anthracene groups (from 1.36 up to 1.44 Å). The framework of the system is ideally in a plane (178.39°) from where each anthracene group deviates 45.84°. These two arms are convertible through a 2-fold symmetry that lies on the central carbon atom (C1) of the molecule. The intramolecular O···H···O angle was calculated as 161° with a theoretical distance O···H of 1.252 Å. Intermolecular interactions appear relevant in the x-direction, within the enol/keto groups of one molecule and the proton from the methine group of the neighbor (O2···H1−C1; with a distance O···H of 2.530 Å and an angle of 150.80°). No π-stacking interactions were found between the aromatic rings (distances above 3.8 Å).

The compound [(phen)CuCl(9Accm)] (**1**) crystallizes in the triclinic space group $P\bar{1}$. Four different, crystallographically independent molecules compose the asymmetric unit (Supporting Information, Figure S3). Selected bond lengths and angles are listed in Supporting Information, Table S2. Each of these 4 molecules exhibits different crystallographic angles and distances, but overall they present identical arrangements, where the copper(II) ion is always pentacoordinated, displaying a distorted square pyramidal geometry (τ values of 0.18, 0.18, 0.13 and 0.09). Figure 2 shows a POVRAY projection for 1 unit of the system. The base of every compound is formed by the two chelating ligands, phenantroline and 9Accm$^-$, meanwhile the Cl$^-$ ion is located on the apex of the pyramid. On average, the Cu atom is located at 0.3 Å above the plane, because of constraints introduced by the chelating ligands. Cu−Cl distances are found 2.4788(10), 2.5019(9), 2.4868(10), and 2.4580(10) Å for Cu1 to Cu4, in that order. Cu−N and Cu−O are between 2.017(3) and 2.049(3) Å and 1.919(2)−1.947(2) Å, respectively. All of them are in agreement with others reported elsewhere.[44,45] All four molecules display similar O−Cu−O angles (between 93.25 and 93.94 Å) that clearly differ from the calculated O−H−O of 161° for the free ligand.

Remarkable are the different conformations displayed for 9Accm upon coordination; this in fact is an excellent demonstration of the flexibility of this anionic ligand. In addition, the opening of the chelating group is quite different from the structure of 9Accm; the angle C1−C3−O2 of the free ligand is 119.99°; this value is close to 125° in compound **1**. A quite complex unit cell is observed,

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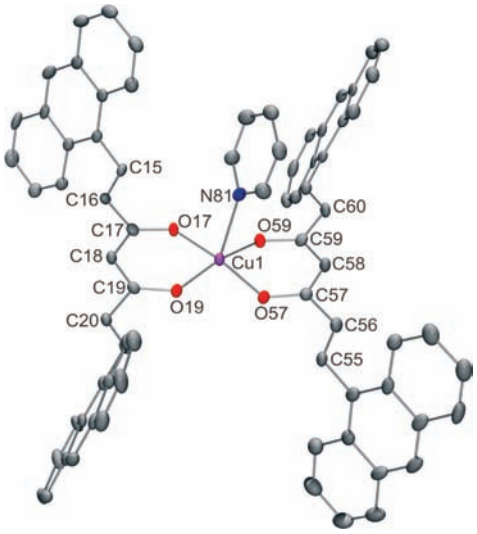

Figure 3. POV-Ray representation of compound **2·py**, [Cu(9Accm)$_2$(py)]. Hydrogen atoms and labels of the anthracene groups have been removed for clarity. Carbon atoms are in gray, oxygen in red, nitrogen in blue and copper in purple colors.

showing hydrogen bonds between water molecules and some of the Cl^- ions, as well as between molecules of water with the non-coordinating solvent, DMF. In addition, weak π-stacking interactions among phenanthroline units and phenanthroline-anthracene groups also exist (Supporting Information, Figure S4).

Compound [Cu(9Accm)$_2$(py)] (**2·py**) is depicted in Figure 3 and relevant bond lengths and angles listed in Supporting Information, Table S3. This compound crystallizes in the triclinic space group $P\bar{1}$. Like the previous structure, compound **2·py** adopts a square pyramidal geometry with the two chelating $9Accm^-$ anions on the base and the pyridine group on the apex of this polyhedron (τ = 0.10).[46] Mainly, symmetry within the molecule is broken because of the twisted coordination between the pyridine molecule and the metal center and also by the different bending angles between the Cu ion and the CO groups of the $9Accm^-$ ligands. The Cu−O distances vary from 1.924(2) to 1.943(2) Å, with a Cu-Npy distance of 2.331(3) Å, being similar to others found in the literature. C−C distances in the backbone and anthracene groups of **2·py** show similar conjugated systems to those found for the free ligand and compound **1** with comparable parameters. On the other hand, the C−C−O angles are smaller than in compound **1**, between 123.88 and 124.72°. No relevant hydrogen bonds or π-stacking interactions were found in the structure.

Fluorescent Studies. Fluorescent studies in air-saturated CH_2Cl_2, DMF, and MeCN solvents were performed to calculate the quantum yield of the samples under study. This way, it was found that the fluorescence excitation and maximum emission, λ_e = 470 nm and λ_f = 580 nm, respectively, were the same for 9Accm and the two copper compounds. The fluorescence quantum yields (Φ_f) of the free ligand in DMF and MeCN were found to be identical, Φ_f = 0.01, and smaller than in CH_2Cl_2, Φ_f = 0.05. Two parallel experiments using 9Accm in dioxygen saturated CH_2Cl_2 and in argon instead, show a decrease of 10% in λ_e intensity for the former (dioxygen is commonly known as a quencher in fluorescent processes). On the other hand, the Φ_f values of **1** and **2** were found analogous; being for both 0.005, 0.002, and 0.003 in CH_2Cl_2, DMF, and MeCN, in that order. Usually, ligands loose their fluorescent properties once coordinated to copper. Here, only a partial quenching is evidenced and significant fluorescence still remains in both complexes.

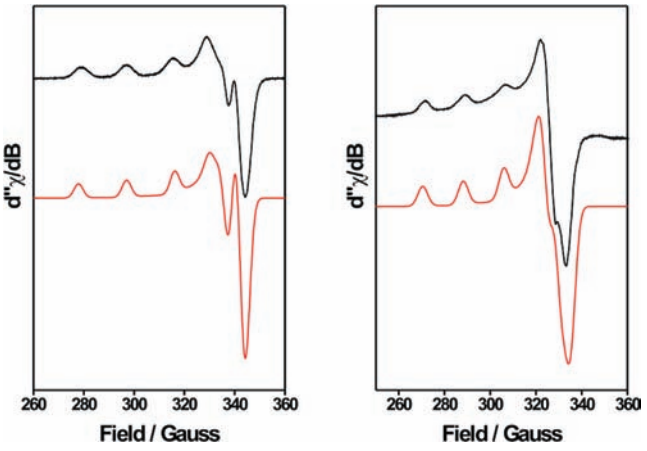

Figure 4. EPR spectra of compounds **1** (right) and **2** (left) in frozen DMF at 70 K. In each graph: experimental data (top) in black and simulation of the data (bottom) below in red.

Magnetic Studies. EPR spectra of the two compounds were measured at different temperatures (298 and 70 K) in the solid state and in frozen solution in DMF (Figure 4) and DMSO (similar, not shown). Experiments agreed with their corresponding crystal structures and proved the stability of the complexes in solution. Both spectra show similar features (black lines) with well-resolved hyperfine lines typical of square planar or square pyramidal Cu systems. Simulation of the experimental data was performed (red lines) giving as a result values of A_x = 10, A_y = 25, and A_z= 220 G, g_x = 2.08, g_y = 2.04, and g_z = 2.26 for **1** and A_x = 15, A_y = 25, and A_z = 220 G with g_x = 2.02, g_y = 2.00, and g_z = 2.20 for **2**. Although these two compounds are strictly rhombic, they could be described as axial systems, where $g_{||}$ is larger than $g_\perp$, indicating elongation of the system (unpaired electron in the $d_{x^2-y^2}$ orbital). The shape and parameters resemble those found for other mononuclear Cu structures with square-planar environments having acac ligands or derivates.[47,48]

The magnetic susceptibility behavior of compounds **1** and **2** was measured using a 1.0 T field in the 2−300 K range (Supporting Information, Figure S5). As expected, the experimental data show a plateau that started at 300 K with values of 0.52 and 0.55 cm^3 mol^{-1} K for **1** and **2**, respectively, until approximately 20 and 8 K for **1** and **2**, respectively, decreasing then to 0.33 and 0.38 cm^3 mol^{-1} K, also in that order. The fitting of the data using Curie's law, corroborated the S = $^1/_2$ ground state for each system, as well g values of 2.15 and 2.06, close to the average of those found with EPR.

Biological and Biophysical Studies

Circular Dichroism. The molecular interactions of compounds **1** and **2** with DNA have been studied using

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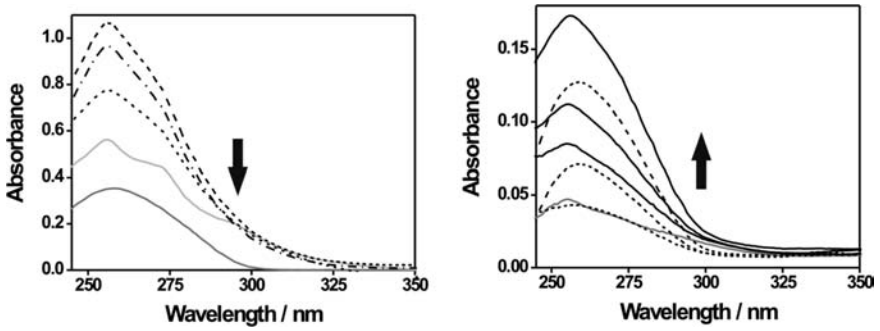

Figure 5. UV−vis titration graphs for compounds **1** (left) and **2** (right). (Left) Dark gray shows the spectrum of free DNA, in light gray the one of the complex by itself and rest of the lines correspond to *R* values of 1.8, 2, and 5 (short-lines, dots/short-lines, and dots respectively). (Right) Gray line indicates the spectrum corresponding to compound **2** alone; black lines are the resulting experiments at $R = 1$, 2, and 5 and the dotted lines are the measures of free DNA at those concentrations. The arrows indicate the direction in which *R* increases. (R = [DNA per nucleotide]/[complex]).

CD spectroscopy (Supporting Information, Figures S6, R = [DNA per nucleotide]/[complex]). Control experiments using calf thymus DNA (right handed B-DNA) were performed in addition to comparative studies with acridine, a well-known DNA-intercalator. Compounds **1** and **2** at various concentrations were incubated with native DNA, and the outcome of their interactions was monitored. From the data it was concluded that their effects clearly differ. On one hand, compound **1** induces a change in the band at 275 nm, which increases, similar to acridine. However, the ecliptic intensity of the negative band also rises, a behavior that varies from acridine and from ethidium bromide too, another common intercalator.[49] This feature has been experienced by others before with diverse interpretations.[50,51] The above effects could on one hand be the result of a conformational change from the initial DNA to a Ψ form, associated with the formation of extended chiral arrays.[52] Alternatively, they could also be described as a modification from a B to an A form of the DNA,[53] according to which there would also be a shift from 275 to 280 nm. The limiting sensitivity at the working concentrations makes both explanations plausible and consequently, additional DNA experiments were performed to further elucidate the nature of this interaction.

On the other hand, compound **2** does not exhibit a pronounced effect on DNA, even not at the highest *R* values, which may be partly due to its poor solubility in the mixture of DMSO (1%):aqueous media. Raising the amount of the organic solvent to 10% (with no drastic changes on the blank, that is, 10% DMSO in aqueous media containing free DNA), did not provide significant variations. Earlier described copper-curcuminoid compounds present similar characteristics with DNA.[54,55]

UV−vis Titration. Experiments with **1** were performed varying the concentration of the compound, while DNA remained constant. On the contrary, because of the low solubility in aqueous media of compound **2**, this amount was kept fixed, and the DNA concentration was modified.

In general, complexes with planar aromatic ligands are expected to interact with DNA through intercalation, exhibiting hypochromism and a red shift in their adsorption bands.[55−58] However, within the 260 to 300 nm range, compound **1** presents hyperchromism. Absorbance bands were found higher than the theoretical sum of the independent absorbances of both, compound and free DNA, and slightly shifted to the red region in the same way as the compound alone (Figure 5, left). Parallel tests performed with compound **2** showed a similar behavior (Figure 5, right). Hence, no obvious intercalation processes were observed for either of the compounds. Kumar et al. experienced before hyperchromism with a copper phenanthroline complex.[59] They concluded that this effect could be the result of hydrogen bonds or electrostatic interaction of the positively charged complex with the negatively charged phosphate backbone of the DNA.[59,60] In addition, Hirohama et al. established that red shifts may be assigned to changes in the coordination sphere of copper compounds resulting from their interaction with DNA.[61] On the other hand, experiments over time for both compounds show a decrease of the absorbance after 2 h, suggesting precipitation of the compounds or chemical adducts formed under these conditions (Supporting Information, Figure S7).

DNA Gel Electrophoresis. These experiments were performed to provide more insight into the nature of compound-DNA interactions and, ultimately, to discard some of the options, as for example cleavage. Precedents are known of copper species able of cleavage of DNA even in the absence of reductant;[62] thus, tests using compounds **1** and **2** were pursued to elucidate their action.

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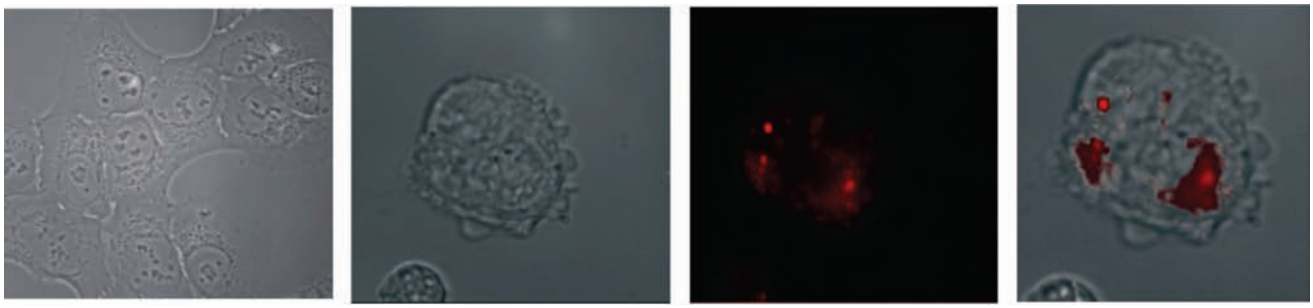

Figure 6. From left to right: (1) U2OS cells in aqueous media before treatment using compound **1**; (2) US2O cell after 1 h of incubation at 37 °C treated with compound **1**; (3) fluorescence image of the treated cell; (4) overlap of pictures 2 on 3 (increased size).

Table 2. IC_{50} Values (μM) of 9Accm, Compounds **1** and **2** with Different Cell Lines[a]

cell lines/compounds	9Accm, μM	**1**, μM	**2**, μM	Cisplatin, μM
A498	80	3	49	7
EVSA-T	70	2	48	1
H226	75	4	48	11
IGROV	> 130	3	> 62	< 1
M19	55	1	40	2
MCF-7	75	4	61	2
WIDR	> 130	3	> 61	3

[a] Results provided by TEVA-Pharmachemie.

The two compounds were measured simultaneously at different concentrations using supercoiled DNA, and studies on cleavage and unwinding processes were carried out for both species.

Cleavage studies in the absence of reductant (Supporting Information, Figure S8) show that no sharp bands caused by circular and linear DNA were detected, suggesting that the two copper compounds do not cleave DNA. Furthermore, the electric current moves negatively charged DNA downward and in the opposite direction of positively charged complexes. On the other hand, the dark stain in the lanes in **2**, which apparently should be neutral, could suggest subsequent reactivity in solution. Additional tests in the presence of reductant provided similar answers to those above (images not shown).

DNA unwinding promoted by **1** and **2** was also investigated (Supporting Information, Figure S9). Intercalation should be identified through a typical smear, instead of well-defined DNA lines.[63] The experiments performed clearly demonstrate that this pattern is not observed for any of the present compounds. This way, we could conclude that neither intercalation nor cleavage are the leading processes in the compound-DNA interactions. All together, these experiments point toward weak, electrostatic bindings between the copper compounds and DNA; together with the titration experiments, the hypothesis of electrostatic interactions rises as one of the most plausible arguments to comprehend DNA-complex behavior.

Cytotoxicity Studies. IC_{50} values for 9Accm and compounds **1** and **2** were determined on different human carcinogenic cell lines as described in the Experimental Section. Cisplatin was used as a reference in all the used cell lines. Experiments of 9Accm with carcinogenic cell lines showed poor cytotoxic activity for the free ligand. In addition, Table 2 displays higher values for **2**, meaning also low cytotoxic effects on carcinogenic cells. However, compound **1** exhibits IC_{50} numbers in the range of cisplatin, being significantly active. So, solubility and stability in biological media must be main factors that could explain the difference between these two compounds (**1** vs **2**). Again, the lability of the Cl^- ions in **1** could facilitate the solubility and enhance the activity of the compound. These results present **1** as a new addition to the noteworthy family of copper compounds with IC_{50} values in the micromolar range.[12,54,64−66]

Fluorescence Studies. Taking advantage of the fluorescence properties observed in compounds **1** and **2**, additional in vitro studies were designed to analyze their path of action. Incubation of these compounds with U2OS cells showed accumulation and fluorescence in vacuoles, outside the nuclei. Nevertheless, as it happened in the previous experiments, the results for both compounds greatly differ. Compound **2** did not show relevant effects in U2OS cells; however, most of the cells treated under similar conditions with **1** experienced apoptosis or necrosis (Figure 6, enlarged version in the Supporting Information), matching once more with the cytotoxic results shown above. This preliminary observation suggests that compound **1** has a different path of action than cisplatin and by some means, agrees well with the weak affinity observed between these species and DNA (experiments above). Even so, the limiting detection of this spectroscopic technique should be taken into account before discarding the possibility of **1** inside the nuclei. Also, the digestion or creation of new adducts (but still fluorescent) inside the cells are questions that remain, and would require additional studies.

Concluding Remarks

In the present work, we have introduced a new curcuminoid ligand, 9Accm, and studied its reactivity as a chelating ligand with copper salts. This way, compounds **1**, [Cu(phen)-Cl(9Accm)], and **2**, [Cu(9Accm)$_2$], have been achieved and their spectroscopic and magnetic characterizations attained. To the best of our knowledge, only one previous ruthenium/curcuminoid compound has been crystallographic reported

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earlier, making compounds **1** and **2** the first two copper/curcuminoid structures described until now in the literature. The crystallographic data show different conformations of 9Accm, in both versions, free and coordinated, stressing the flexibility of the diarylheptanoid system with changes in the opening of the β-diketone group and anthracene arms dispositions. Compounds **1** and **2** possess pentacoordinated copper(II) centers, where each of these metallic species displays a distorted, square-planar pyramidal geometry, with a Cl anion (compound **1**) or a pyridine molecule (**2**) occupying the apex of such polyhedrons. Magnetic studies are consistent with these descriptions and agree with those of other copper compounds having $acac^-$ or dbm^- as ligands. Additional studies in solution have portrayed the fluorescent behavior of 9Accm, as well as that of the two metal compounds; as expected, these latter present reduction of the fluorescence intensities because of coordination of the ligand with copper.

Owing to the biomedical interest that a number of copper compounds and curcuminoids have roused in the scientific community, biophysical studies, such as CD, UV−vis titration, and gel-electrophoresis, were performed with [Cu(phen)Cl(9Accm)] and [Cu(9Accm)$_2$], respectively. Originally, because of the planar and aromatic nature of the ligands, the reversible inclusion of these compounds within DNA was considered as one of the most reliable ways of interaction. However, all the experiments performed agree with a lack of intercalation between the anthracene groups (or phenantroline) and the DNA. In fact the outcome of these tests rather points instead toward weak, electrostatic interactions. At the same time, the cytotoxic activity of **1** and **2** was tested; showing high activity of compound **1** (in the same range or even better than cisplatin) against a variety of carcinogenic cell lines, whereas compound **2** exhibits hardly any activity. The difference between these two compounds may arise from the ability of **1** to exchange the chloride ion with the media, becoming charged, more soluble, and therefore prone to react. On the other hand, in vitro trials carried out using fluorescent microscopy indicate accumulation of both compounds in vacuoles outside the nuclei, suggesting that perhaps DNA may not be the primary target of compound **1** and thus, that its activity may involve additional or completely different types of reactions, once these species are inside the cells; therefore, this could lead to a completely new class of metal-anticancer drugs.

Acknowledgment. The authors thank the Ministerio de Educación y Ciencia (CTQ2009-06959/BQU) and ICREA (Institut Català de Recerca i Estudis Avançats) for the financial support. The cytotoxicity tests of the compounds were generously supported by Dr. D. de Vos from Teva/pharmachemie, B.V, The Netherlands. We acknowledge the provision of time at the CCLRC Daresbury Laboratory, SRS, via support by the European Union. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

Supporting Information Available: Tables S1, S2, and S3 with crystallographic details of 9Accm and compounds **1** and **2·py**; UV−vis spectra of free 9Accm, **1** and **2** in DMSO and DMSO+buffer; crystallographic packing diagram of compound **1** and also packing diagrams showing intermolecular interactions for the same compound; magnetic susceptibility data, and fitting results, CD spectra, UV−vis titration spectra, and gel-electrophoresis experiments of **1** and **2**, respectively. This material is available free of charge via the Internet at http://pubs.acs.org. Crystallographic data files (cif format) for 9Accm and compounds **1** and **2** have been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition numbers CCDC 775933−775935.