Structural Elucidation of Cisplatin and Hydrated cis-Diammineplatinum(II) Complex Conjugated with Cyanocobalamin by Liquid Chromatography with Electrospray Ionization–Mass Spectrometry and Multistage Mass Spectrometry

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 Supporting Information

ABSTRACT: Pt(II)-based derivatives bearing a cyanocobalamin (CNCbl) unit were synthesized in aqueous solutions, and the reaction mixtures were examined by reversed-phase liquid chromatography with electrospray ionization and linear ion trap mass spectrometry (MS). Isotopic pattern analysis, multistage mass-spectra (MS/MS and MS3) interpretation, and differential isotopic labeling were used to establish the chemical composition and to suggest the chemical structures of reaction products. When cisplatin (cis-[PtCl2(NH3)2]) was used as a Pt(II) drug derivative, a coordination bond between diamminemonochloroplatinum(II) and the cyano group of CNCbl, in turn linked covalently to the vitamin Co(III) ion, occurred. The resulting conjugate with a CoIII–CN–PtII bridge was MS detected as a doubly positive charged ion with the prevailing isotopologue at m/z 810.26 (empirical formula [C63H95ClCoIIIN16O14PPt]2+). Likewise, a peak signal centered at m/z 811.26 was observed when 15N-labeled cisplatin cis-[PtCl2(15NH3)2] was used as Pt(II) complex, thus confirming the presence of both the cisplatin amino groups in the conjugate. A bifunctional conjugate was obtained between CNCbl and the cis-diamminediachloroplatinum(II), that is, cis-[Pt(NH3)2(H2O)2]2+, in this case, the planar coordination complex of Pt(II) was also involved in a covalent bond with the oxygen atom of one of the CNCbl amide moieties. The peak signal detected at m/z 792.26 (empirical formula [C63H94CoIIIN16O14PPt]2+) changed to m/z 793.26 when the labeled cis-[Pt(15NH3)(H2O)2]2+ complex was adopted for conjugation. Comparison between MS/MS spectra allowed an extended structural characterization of both conjugates, as such or 15N-labeled. Two-dimensional heteronuclear (1H–15N) single quantum correlation NMR spectroscopy, applied to 15N-labeled conjugates, supported the hypotheses made on the Pt(II) coordination in both cases.

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INTRODUCTION

Cisplatin (CP), cis-diaminedichloroplatinum(II), is a widely used chemotherapeutic agent in the treatment of human malignancies but exhibits low selectivity for tumor tissue, which leads to severe side effects.1−5 Continuing research is uncovering new beneficial forms of platinum-based drugs,6,7 leading to so-called prodrugs.8−10 Cyanocobalamin (CNCbl or vitamin B12) uptake in mammalian cells is mediated by specific, high-affinity receptors that are overexpressed in numerous human tumors.11 The affinity of CNCbl conjugates for such receptors remains high enough, and rapidly proliferating tumor cells ask for increased supply of nutrients, including CNCbl. As a consequence, CNCbl–CP conjugates can be considered interesting candidates as Pt(II)-based prodrugs. Cellular uptake of CNCbl–CP conjugates is typically followed via detection of their metal constituents by inductively coupled plasma mass spectrometry (ICP–MS), a powerful technique that, however, lacks speciation capability.7,8 Investigation of prodrug metabolism would further benefit from information at molecular level that can be gathered by, for example, soft-ionization MS techniques. Indeed, matrix-assisted laser desorption ionization (MALDI)−MS has been recently proposed for the structural characterization of CNCbl and CNCbl–Pt(II) species (e.g., CP and oxaliplatin) conjugates.9 Using a second-generation MALDI matrix, it was demonstrated that CNCbl–CP gives rise to a single conjugate involving a CN bridge between Co(III) and Pt(II), whereas two conjugates were obtained for oxaliplatin, differing from CP for the replacement of the two amine and both Cl− ligands by...
the bidentate ligands 1,2-diaminocyclohexane and oxalate, respectively.

Cyanocobalamin’s structure comprises a mid-planar corrin ring composed of four pyrrole rings linked to a central Co(III) ion. CNCbl is essential for the survival of all living organisms playing a key role in the hematopoiesis, neural metabolism, DNA and RNA production, and carbohydrate, fat, and protein metabolism.10,11 More than 10 years ago, Alberto and co-workers proposed the idea to use CNCbl as a carrier of CP;12−14 it was shown that CNCbl can act as a ligand for CP by formation of a cyanide bridged species between the β-axial ligand of CNCbl and the square-planar platinum complex.12 The CNCbl−CP conjugate retains a labile chloride ligand that can be exchanged within cells, allowing the conjugate to behave in a comparable way to CP. Preliminary results showed a lower activity (IC50 between 8 and 88 µM) than for CP alone, possibly because of limited CNCbl uptake capacity. Because platinum-based drugs interact with DNA15 and many other biomolecules,16−17 a full understanding of their modes of action requires the knowledge of all species formed during the reaction process of CP and CNCbl. Yet, few detailed mechanistic studies have been performed on such interactions, in comparison to studies with nucleobases. Accordingly, understanding the binding mechanism of CP to CNCbl is of highest importance for the development of refined therapeutic approaches of drug delivery. Several analytical techniques are currently available for monitoring CP uptake and formation of Pt−DNA adducts in biological samples, mostly relying on ICP−MS.18 Concomitantly, good sensitivity and excellent selectivity is required to advance the understanding of drug Pt-based cytotoxicities and to further elucidate the cell uptake, resistance, and treatment monitoring.

Soft ionization MS techniques, such as electrospray ionization (ESI), which contrary to MALDI can be easily coupled on-line to liquid chromatography (LC), deserve further investigation, aimed at analyzing platinum prodrugs metabolism in complex biological fluids or tissues. Although LC−ESI−MS methods for CNCbl determination in complex samples (especially foods) have been reported, no study exists concerning simultaneous determination of CNCbl and CNCbl−Pt(II) drug conjugates. In the present study, the characterization of two distinct types of complex [i.e., cis-diamminedichloroplatinum(II) and its hydrated cis-diammineplatinum(II)] conjugates with CNCbl is presented, mainly based on reversed-phase LC (RPLC) coupled with ESI and collision-induced dissociation (CID) multistage MS. A confirmation of the proposed structures was obtained through two-dimensional heteronuclear correlation NMR spectroscopy (widely used in anticancer platinum drugs study19), which also allowed to discern between equivalent structures from the point of view of the MS and MS/MS spectra. In this synergistic approach, MS data were used to obtain the elemental composition of each conjectured adduct; CID−MS/MS spectra collected on isotopically labeled Pt(II) complexes with 15NH3 ammonia molecules lead to noticeable benefits for structure determination, with further refinements by NMR analysis.

RESULTS AND DISCUSSION

RPLC−ESI−MS of CNCbl Reaction Mixtures with CP or 15N-Labeled-CP. The RPLC−ESI−MS total ion current chromatograms, referred to the reaction mixtures containing either CNCbl plus CP (i.e., cis-[Pt(NH3)2Cl2]) or CNCbl plus hydrated cis-diammineplatinum(II) (i.e., [Pt-(NH3)2(H2O)2]2+),20 are reported in plots a and b of Figure 1, respectively. A comparison of the chromatographic profiles reveals the occurrence of two common peaks, labeled as 1 and 3 (see plots a and b), with retention times 11.6 and 9.3 min, respectively. The most intense signals of the corresponding ESI−MS spectra were detected at m/z 678.30 and 792.29 (vide infra). Regarding peak 2, detected only in plot a and eluting at 9.6 min, the most intense peak of the isotopic cluster was found at m/z 810.28. Peak 1 was assigned to CNCbl by a standard solution examined under the same LC−MS conditions as emphasized in Figure S1 (Supporting Information). The major signal at m/z 678.30 in the ESI−MS spectrum is was due to a doubly charged ion corresponding to the bis-protonated form of zwiterionic CNCbl (i.e., [C63H88CoN14O14P + 2H]2+). Two additional ion clusters, with most intense peaks detected at m/z 689.28 and m/z 697.27, respectively, were recognized as doubly charged ions resulting from the previous species through the exchange of a proton with a sodium ([M + H + Na]2+) or a potassium ([M + H + K]2+) cation, respectively (with M representing zwiterionic CNCbl). Under the present LC−MS conditions, the peak due to mono-protonated CNCbl ([M + H]−), detected at m/z 1355.57, was very weak, its relative abundance being ca. 1% of that of the bisprotonated molecule (see Figure S1). Notably, the same signal was detected as the most abundant one during a previous investigation based on MALDI−ToF−MS using 4-chloro-α-cyanoacinnamic acid as a matrix.21

The major signal (m/z 810.28) detected in the mass spectrum related to peak 2 of Figure 1a (i.e., the spectrum reported in Figure 2a) readily suggested the occurrence of a molecular species containing platinum, which is characterized...
When 15N-labeled CP (i.e., cis-diaminedi-aqua-Pt(II) complex, corresponding to peaks (2) and (3) in Figure 1, respectively. Insets show the enlarged isotopic patterns located at (a) m/z 810.28 and (b) m/z 792.29 compared with isotopic patterns simulated using the reported empirical formulae and a resolving power 4000 (see boxed frames). Xcalibur software 2.2 SP1.48 (Thermo Scientific) was used to simulate the isotopic patterns.

Starting from the outcomes reported so far, including empirical formulas, and considering the formation of a CN bridge between CoIII and PtII (i.e., CoIII–CN–PtII) as previously suggested, the chemical structures proposed for CNCbl–CP conjugates whose diagnostic ions were detected at m/z 810.3 (structure 1) and 792.3 (structure 2) are depicted, in a simplified form, in Figure 3, including the corresponding 15N-labeled conjugates (1′ and 2′). The structures of non-labeled conjugates are reported in Figure S3 (Supporting Information). The experimental m/z values of major peak signals related to unlabeled (m/z 810.28 and m/z 792.29) and 15N-labeled adducts with CNCbl (m/z 811.28 and m/z 793.29, see Figure S2) agreed with the predicted values of the corresponding ion clusters, viz., 810.2809, 792.2930, and 793.2901, respectively. Although there is a good accordance between structure 1 in Figure 3 and the one proposed by Ruiz-Sánchez et al.13 for the CNCbl–CP conjugate, based on X-ray diffraction data, no information is available in the literature on the occurrence of an additional conjugate (see structure 2 in Figure 3) detected by the presence of major natural isotopes 194Pt(32.9), 195Pt(33.8), 196Pt(25.3), 198Pt(7.2), and 192Pt(0.8), here listed in a decreasing order of percentage abundance (reported in parentheses).22 As apparent in the enlarged view reported in Figure S2a, the isotopic pattern, dominated by a signal at m/z 810.2809, clearly corresponded to a doubly charged ion and was found to agree with a simulated spectrum (see Figure S2a inset) based on the empirical formula [C_{63}H_{95}ClCo^{III}N_{16}O_{14}Pt^{II}]^{2+}. Obviously, the presence of chloride increases the isotopic pattern complexity of the ion cluster centered at m/z 810.28. When 15N-labeled CP (i.e., cis-[PtCl(_2)(15NH_3)_2]) was incubated with CNCbl, a peak eluting almost at the same retention time (i.e., 9.6 min) of the unlabeled adduct was observed. The corresponding ESI–MS spectrum, reported in Figure S2 of the Supporting Information, exhibited the most intense peak at m/z 811.28 and the related isotopic pattern was in excellent agreement with the simulated pattern obtained using the empirical formula [C_{63}H_{94}Co^{III}N_{16}O_{14}Pt^{II}]^{2+}; that is, a formula arising from the previous one upon replacement of two 14N atoms with two 15N ones. This result confirmed that both amino groups of CP were involved in the structure of the compound corresponding to peak 2, whose empirical formula agrees with data of Alberto and co-workers,12,13 and the generation of a conjugate with CNCbl, likely implying the presence of a Co–CN–Pt bridge (vide infra).

Turning to peak 3, detected in both chromatograms of Figure 1 (although its amount was significantly different in the two cases), an ion cluster with a major peak at m/z 792.29 was found in the corresponding ESI–MS spectrum, reported in Figure 2b. The corresponding isotopic pattern was found to agree with [C_{63}H_{94}Co^{III}N_{16}O_{14}Pt^{II}]^{2+} as empirical formula (see inset of Figure 2b), in which a different conjugation between CNCbl and cis-diamino-Pt(II) is invoked, with a formal loss of HCl compared to the conjugate formed between CNCbl and CP. Interestingly, the isotope pattern related to the main signal detected in the ESI–MS spectrum of Figure 2b, corresponding to a m/z 664.76 ion, suggests the absence of platinum in the corresponding ion, whose identity will be discussed below. By analogy with the conjugate eluted as peak 2, a chromatographic peak at the same retention time as peak 3 in Figure 1 was observed upon the analysis of a reaction mixture containing CNCbl and the 15N-labeled cis-diammineplatinum(II) complex. An ion cluster dominated by a m/z 793.29 ion, corresponding to the empirical formula [C_{63}H_{94}Co^{III}N_{16}O_{14}Pt^{II}]^{2+}, was found in the corresponding ESI–MS spectrum (see Figure S2b, Supporting Information).

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in the present case, that is, the one lacking chlorine ([C₆H₆Co[N₆O₄]PPt][²]). As emphasized by Figure 1, this conjugate was virtually the only one generated in the reaction mixture when the hydrated cis-diamineplatinum(II) complex was used as a CNCbl reagent. This finding supports the hypothesis made for structure 2. Indeed, the complete lack of chloride ligands in the reacting Pt(II) complex obviously favors the generation of a CI-free conjugate with CNCbl, which in turn enhances the occurrence of two bonding sites of the planar Pt(II). One of such bonds is still related to the CN group of CNCbl; the other is reasonably related to one of the CNCbl amide arms, that is, the deprotonated OH moiety included in the enol-iminic tautomer of the corresponding amide group (vide infra). Notably, the generation of a CI-free conjugate is possible also when CP is used as a conjugation reagent, yet, as expected, it is much more difficult, because both of the chloride ligands of CP must be replaced. Compelling evidence is given by the very low abundance of the chloride ligands of CP, which resonate approximately between -70 and -90 ppm when 15N is trans to O and between -50 and -70 ppm when 15N is trans to Cl or N, and were extremely diagnostic. The 1H-15N HSQC spectra of CP, incubated under the same experimental conditions of the mixture, displayed the signals related to the di-chloro species (cis-[PtCl₂(15NH₃)₂], Δ₁5N = -67 ppm), the mono-chloro mono-aqua species (cis-[PtCl(15NH₃)(H₂O)], Δ₁5N = -65 and -88 ppm for the 15NH₃ ligand trans to Cl and trans to O, respectively), the hydrated complex (cis-[Pt(15NH₃)₂(H₂O)]²⁺, Δ₁5N = -84 ppm), and other signals assigned to hydroxo-bridged complexes. After 16 h of reaction with CNCbl, in addition to the previously described NMR signals, 15N-CP displayed two intense signals, falling in the region of 15N trans to Cl or N (Δ₁5N = -66 and -68 ppm) and confidently assigned to the adduct with CNCbl.

The reaction with CNCbl was also carried out with cis-[Pt(15NH₃)₂(H₂O) (SO₄)], which generates the hydrated species cis-[Pt(15NH₃)₂(H₂O)]²⁺ (Δ₁5N = -84 ppm) after dissolution, thus mimicking the intracellular active form of the drug. The presence of residual signals of the mono-chloro mono-aqua ligands (Δ₁5N = -65 and -88 ppm) was ascribed to Cl contamination during the synthesis of the complex. After 16 h of incubation with CNCbl in water (pH 3.6) at 60 °C, new signals appeared in the 1H-15N HSQC spectrum (Figure 4b). The two most abundant peaks were assigned to 15NH₃ ligands trans to O and trans to N, respectively (Δ₁5N = -76 ppm and -61 ppm). This finding strengthened the hypothesis made on the proximity of an oxygen atom to the Pt(II) one in the conjugate generated when the cis-[Pt(15NH₃)₂(H₂O)]²⁺ complex is reacted with CNCbl.

Structural Characterization of CNCbl-Pt(II) Conjugates by CID Tandem MS (CID-MS/MS). Taking advantage of the information obtained by MS and NMR analysis, the conjugates formed between CNCbl and Pt(II) complexes were further investigated by tandem MS with CID (CID-MS/MS). Figure 5 (plots a–d) shows the MS/MS spectra obtained for the corresponding precursor ions, including also those related to ₁⁵N-labeled conjugates (thus isotopic patterns centered on nominal m/z ratios 810, 811, 792, and 793 were considered for MS/MS analyses). Each of the main product ions detected was carefully evaluated in terms of m/z ratio, charge state, and isotopic pattern, and the corresponding empirical formula was obtained; then the corresponding structure or the neutral loss/losses leading to that formula were assumed. The assignments made at the end of this systematic procedure for product ions related to both non-₁⁵N-labeled conjugates under study have been summarized in Tables 1 and 2.

At first glance, several signals related to common product ions can be observed in the MS/MS spectra of Figure 5 (plots a–d). One of them is detected at m/z 359.09 and 341.09, clearly not containing Pt in their structures and easily assigned to the protonated form of the so-called nucleotide loop (NL) of CNCbl, that is, of α-5,6-dimethylbenzimidazole-ribosyl-5'-phosphate (see Figure S4), and to the ion arising from it.
upon water neutral loss, respectively. Among further common product ions generated in the gas-phase, the doubly charged one detected at \( m/z \) 635.76 for the CNCbl\(^-\)CP conjugate can be explained by assuming the neutral losses of an acetamide radical (\( \cdot \text{CH}_2\text{CONH}_2 \)), from one of the amide arms of CNCbl, and of the neutral Pt(NH\(_3\))\(_2\)ClCN complex. Although well defined, the loss of the acetamide radical, clearly leading to radical ions as fragments, was involved in several fragmentation pathways of the conjugates under study, as emphasized in Tables 1 and 2. A different pathway had to be proposed for the \( m/z \) 635.72 ion obtained upon fragmentation of the conjugate formed by CNCbl and the hydrated cis-diammineplatinum(II) complex. As depicted in Figure S5, the product ion resulted from the direct detachment of the Pt(NH\(_3\))\(_2\)CN(O(C\(\equiv\)NH)CH\(_3\))\(^-\) neutral complex, implying, once again, the homolytic cleavage of the bond linking the corrin ring to the CNCbl amide arm involved in the Pt(II) complexation. In this case the final double positive charge was located on the Co(III) ion. As an alternative pathway involving one of the CNCbl amide arms, neutral acetaimide could be lost, through a 1,3-H migration from the corrin ring toward one of the amide arms, generating a product ion at \( m/z \) 635.27 (see Figure 5).

This process was involved also in the generation of the singly charged ion detected at \( m/z \) 912.43 when the CNCbl–CP conjugate was fragmented. In this case the acetamide loss was accompanied by the detachment of the singly charged [Pt(NH\(_3\))\(_2\)Cl]\(^-\) complex and, interestingly, of a CN\(^-\) radical, with the consequent reduction of Co(III) to Co(II) (see Table 1). Notably, the existence of this product ion was proved earlier by MALDI–MS/MS analysis of CNCbl.\(^{21}\) When the conjugate between CNCbl and the hydrated cis-diammineplatinum(II) complex, that is, the right structure in Figures 3 and S3, was fragmented, both NL and CN\(^-\) radical losses had still to be invoked to explain the gas-phase generation of the product ion at \( m/z \) 912.44. However, in this case they were accompanied by the loss of the singly charged [Pt(NH\(_3\))\(_2\)O(C\(\equiv\)NH)CH\(_3\)]\(^+\) species, that is, a complex in which Pt was covalently linked to the O atom related to the CNCbl amide arm previously involved in the conjugation (see structures 2/2′ in Figure 3) and detached from the corrin ring during the fragmentation process.

A further interesting product ion was the \( m/z \) 911.43–911.44 one, whose abundance was ca. 15% of that of the \( m/z \) 912.43 ion in the case of the CNCbl–CP conjugates (see Figure 5).

**Figure 5.** ESI–CID–MS/MS spectra obtained for conjugates formed through reaction of CNCbl with: (a) cis-[PtCl\(_2\)(NH\(_3\))\(_2\)] (nominal \( m/z \) 810), (b) cis-[PtCl\(_2\)(\(^{15}\)NH\(_3\))\(_2\)] (nominal \( m/z \) 811), (c) cis-[Pt(NH\(_3\))\(_2\)(H\(_2\)O)\(_2\)]\(^{2+}\) (nominal \( m/z \) 792), and (d) cis-[Pt\(^{15}\)NH\(_3\))\(_2\)(H\(_2\)O)\(_2\)]\(^{2+}\) (nominal \( m/z \) 793). The prevailing peak of each isotope pattern has been labeled with the \( m/z \) ratio. A detailed assignment of all identified peaks is given in Tables 1 and 2. See text for details.
Table 1. Proposed Structures and Losses Related to Product Ions Obtained by CID–MS/MS for the Conjugate Formed between CNCbl and CP, cis-Diamminedicloroplatinum(II): Precursor Ion [C₆H₅CoIII(N)₁₀O₇P, 358.093 Da], Detected at Nominal m/z 810, See Plot a of Figure 5

| product ions | suggested loss | theoretical m/z | m/z* | relative intensity % |
|--------------|----------------|-----------------|------|-----------------------|
| [C₁₄H₁₉N₂O₇P]⁺ | see Figure S4, H₂O | 810.280 | 810.26 | | |
| [C₁₄H₁₉N₂O₇P]⁺ | see Figure S4 | 341.090 | 341.09 | 8 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | Pt(NH₃)₂CN, NL, CH₃CONH₂ | 359.100 | 359.09 | 56 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, Pt(NH₃)₂CN | 456.724 | 456.67 | 24 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | *CH₃CONH₂, Pt(NH₃)₂CN | 635.267 | 635.27 | 35 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | 635.771 | 635.76 | 100 |
| [C₁₄H₁₈N₂O₆P⁺ see Figure S4 | 664.785 | 664.68 | 9 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | see Figure S4 | 728.725 | 728.73 | 9 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | DMB, NH₃ | 737.238 | 737.23 | 28 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, CH₃CONH₂, 2NH₃ | 745.748 | 745.75 | 10⁶ |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, *CH₃CONH₂, 2NH₃ | 746.252 | 746.25 | 7⁷ |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, CH₃CONH₂, NH₃ | 754.735 | 754.74 | 8 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | *CH₃CONH₂, 2NH₃ | 764.240 | 764.24 | 52 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, 3NH₃ | 766.751 | 766.68 | 78 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, NH₃ | 772.249 | 772.18 | 17 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, 2NH₃ | 774.677 | 774.68 | 20 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | HCl, NH₃ | 783.780 | 783.78 | 20 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | 2NH₃ | 792.792 | 792.79 | 24 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | [Pt(NH₃)₂(O(C₄H₉)C₆H₅)⁺, CH₃CONH₂, Pt(NH₃)₂CN, NL | 911.440 | 911.43 | 13⁶ |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | [Pt(NH₃)₂(O(C₄H₉)C₆H₅)⁺, CH₃CONH₂, CN*, NL | 912.440 | 912.43 | 75 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | [Pt(NH₃)₂(O(C₄H₉)C₆H₅)⁺, HCN, NL | 970.470 | 970.47 | 21 |
| [C₁₄H₁₈CoIII(N)₁₀O₇P]⁺ | DMB, CH₃CONH₂, [Pt(NH₃)₂(O(C₄H₉)C₆H₅)]⁺, CN* | 1124.450 | 1124.45 | 17 |

“The reported m/z ratios are referred to the prevailing isotopologue in the isotope pattern. Ion intensities observed in Figure 5 are because of overlapping of different fragments. Abbreviations: DMB, 5,6-dimethylbenzimidazole base (C₅H₅N₂, 146.084 Da); NL, nucleotide loop (C₁₄H₁₉N₂O₇P, 358.093 Da).”

Table 2. Proposed Structures and Losses Related to Product Ions Obtained by CID–MS/MS for the Conjugate Formed between CNCbl and the Hydrated cis-Diamminedichloroplatinum(II) complex (Precursor ion [C₆H₅CoIII(N)₁₀O₇P⁺, 358.093 Da], Detected at Nominal m/z 792, See Plot c of Figure 5)

| product ions | suggested loss | theoretical m/z | m/z* | relative intensity % |
|--------------|----------------|-----------------|------|-----------------------|
| [C₁₄H₁₉N₂O₇P]⁺ | see Figure S4 and H₂O loss | 792.290 | 792.29 | | |
| [C₁₄H₁₉N₂O₇P]⁺ | see Figure S4 | 341.090 | 341.09 | 19 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH--Pt(NH₃)₂CN | 359.100 | 359.09 | 75 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | Pt(NH₃)₂CN(O(C═NH)CH₂*) | 635.267 | 635.27 | 30⁶ |
| [C₁₄H₁₉N₂O₇P]⁺ | see Scheme S1 | 635.771 | 635.72 | 20⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | DMB, NH₃ | 655.116 | 655.10 | 18 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, CN*, 2NH₃ | 709.745 | 709.75 | 9 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, 2NH₃ | 732.746 | 732.76 | 19 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, NH₃ | 745.748 | 745.73 | 25⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | *CH₃CONH₂, 2NH₃ | 746.252 | 746.25 | 7⁷ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, NH₃ | 754.261 | 754.25 | 60⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | *CH₃CONH₂, NH₃ | 754.765 | 754.68 | 35⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | 3NH₃ | 766.751 | 766.72 | 49 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | 2NH₃ | 774.776 | 774.72 | 100 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | [Pt(NH₃)₂(O(C₄H₉)C₆H₅)⁺, CH₃CONH₂, NL | 911.440 | 911.44 | 36⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | [Pt(NH₃)₂(O(C₄H₉)C₆H₅)⁺, CN*, NL | 912.440 | 912.44 | 30⁶ |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | CH₃CONH₂, CN*, NL, 2NH₃ | 1106.399 | 1106.40 | 10 |
| [C₁₄H₁₉CoIII(N)₁₀O₇P]⁺ | NL, NH₃, NH₃* | 1190.431 | 1190.43 | 22 |

“The reported m/z ratios are referred to the prevailing isotopologue in the isotope pattern. Ion intensities observed in Figure 5 are because of overlapping of different fragments. Abbreviations: DMB, 5,6-dimethylbenzimidazole base (C₅H₅N₂, 146.084 Da); NL, nucleotide loop (C₁₄H₁₉N₂O₇P, 358.093 Da).”

Figure 5a,b), whereas it became comparable in the case of the other two conjugates (see Figure 5c,d). Once again, the structural interpretation for this product ion was different according to the precursor ion. In the case of the CNCbl–CP conjugate, an additional loss of a H⁺ radical, with respect to the pathway leading to the m/z 912.43 ion, had to be invoked, thus
leading to a radical ion (see Table 1). Not surprisingly, this was a difficult process, as confirmed by the weak signal detected for the resulting fragment. When the other conjugate was considered, the pathway involved losses of both acetamide, NL and [Pt(NH3)2CN]2+ ion. As emphasized in Figure S6, no change in oxidation state was required for the Co ion in this case, yet its residual double positive charge was partly compensated by the negative charge located on the oxygen atom previously interacting with Pt(II). The formation of a Co–O covalent bond cannot be ruled out in this case.

As shown in Figure 5, several signals were detected in the 720−800 m/z range of conjugate MS/MS spectra, and, again, part of them were common to both conjugates; moreover, because of their particular isotope patterns, the signals were related to doubly charged ions, including the Pt(II). Their generation implied combinations of processes like the already cited neutral losses of acetamide or of its radical species along with losses of HCl, NH3, S6-dimethyl-benzimidazole (DBM), and H2O (from the ribose ring of the NL). For the sake of example, the pathways leading to product ions at m/z 764.24 and 766.75 from the CNCbl−CP conjugate have been depicted in Scheme 1, including the simplified structure of a planar Pt(II) conjugate. As apparent in the lower pathway of the scheme, the HCl loss from the latter implies the generation of a Pt–O bond like that hypothesized for the conjugate formed between CNCbl and the hydrated cis-diammineplatinum(II) complex. It is then not surprising that the m/z 766.75 product ion was observed also in the MS/MS spectrum of the latter. In the same pathway of Scheme 1, the loss of a further NH3 molecule besides those interacting with Pt, from one of the amide arms of CNCbl (with generation of a ketene), is evidenced.

As expected (see Scheme 1), fragmentation pathways involving the loss of both of the NH3 molecules interacting with Pt(II) led to identical product ions for the nonlabeled and the 15N-labeled conjugate of the same type, because the 15N atoms were present only in the two NH3 ligands. Consequently, a systematic comparison of m/z ratios observed in MS/MS spectra of Figure 5a/b,c/d enabled a rapid assignment of product ions whose generation involved the loss of one or both of the NH3 ligands of Pt(II), always confirming the suggestions made from MS/MS data referred to non-15N-labeled conjugates.

The last considerations on MS/MS data are deserved by product ions with m/z 664.68 and 655.10 (656.11), detected solely from precursor ions at nominal m/z 810/811 and 792(793), respectively. As emphasized in Scheme S1 (top) of the Supporting Information, the first product ion was generated by the loss of the neutral Pt(NH3)2CICN complex, thus leaving a double positive charge on the Co(III) atom of CNCbl. At least formally the product ion can be indicated as a [M−CN]2+, where M represents the zwitterionic form of CNCbl. The fragmentation pathway leading to product ion at m/z 655.10 (656.11) implied the final interaction between the Pt(II) atom and the NL of CNCbl (see the lower side of Scheme S1). In addition, a Pt–CN bond was formed, along with the one between Pt and the O atom of the tautomized amide group of CNCbl, although the amide arm interacting with Pt was detached from the corrin ring of CNCbl. The positive charge of the product ion was thus likely related to protonation of the benzimidazole, as shown in Scheme S1. A single ammonia ligand remained on the product ion structure, thus explaining the 1 m/z unit shift observed when the 15N-labeled conjugate was fragmented.

Finally, the systematic interpretation of MS/MS data provided a confirmation of the structures hypothesized for the two conjugates under study and reported in Figures 3 and S3. Nonetheless, their structural characterization was completed by considering two of the already discussed product ions, those at nominal m/z 764 and 766, for MS3 measurements and is described in the Supporting Information.

## CONCLUSIONS

RPLC−ESI−MS and MS’ (with n = 2 or 3) were successfully applied to establish the chemical identity of two conjugates formed in aqueous solutions between CP and CNCbl and hydrated cis-diammineplatinum(II) and CNCbl. In the former case the generation of a conjugate involving a chloride ligand loss and a coordination bond between the CN group of CNCbl and the Pt ion of CP was observed. The lack of chloride ligands in the cis-diammineplatinum(II) complex led to a different conjugation, still implying a Pt–CN coordination bond along with the formation of a covalent Pt–O bond involving one amide arm of CNCbl. Two-dimensional heteronuclear correlation NMR spectroscopy, performed on conjugates generated under the same reaction conditions but using 15N-labeled Pt(II) complexes as reactants with CNCbl, confirmed the presence of a Cl or an O atom near the Pt atom in both conjugates, respectively. Given the importance of prodrugs to overcome CP resistance, the characterization of conjugates between CNCbl and Pt(II) complexes described here by RPLC−ESI−MS and MS/MS may represent an important contribution to understand the chemical structure of expected and even unexpected complexes containing platinum(II) compounds.
MATERIALS AND METHODS

Chemicals and Synthesis of Pt(II) Complexes. Water and methanol, both LC–MS grade, and CNCbl and formic acid (reagent grade) were obtained from Sigma-Aldrich (Milan, Italy). cis-[PtCl₂{(15)NH₃}₂] and cis-[Pt{(15)NH₃}₂(H₂O)−(SO₄)] were prepared by a slightly modified literature procedure.²⁰ Briefly, K₂PtCl₄ (0.516 mmol) and solid KI (0.720 g, 4.33 mmol) were mixed in water (2 mL) at 55 °C; in a few minutes the solution turned from red to brown. 2 mL of a 6.2 mM aqueous solution of ammonium chloride (or (15)NH₄Cl, in the case of (15)N-labeled CP synthesis) was neutralized with 2 mL of an aqueous solution of KOH 6.2 M and added, under stirring, to the previous solution. The heating was prolonged for 15 min; meanwhile a golden-yellow precipitate separated out. The solid was collected on a filter glass, washed with water and ethanol, and then dried in a stream of dry air. The newly formed cis-[PtI₂{(15)NH₃}₂] (0.190 g, 0.4 mmol) was suspended in water (2 mL). To this suspension, Ag₂SO₄ (0.130 g, 0.4 mmol) was added under stirring at 44 °C, and the reaction was carried out overnight in the dark. The suspension was filtered to remove AgI, and the filtrate was concentrated with a rotary evaporator and subsequently dried. The crystalline solid has the formula cis-[Pt{(15)NH₃}₂(H₂O)−(SO₄)]. To obtain (15)N-labeled CP, solid KCl (0.32 g, 4.33 mmol) was added to the filtrate containing cis-[Pt{(15)NH₃}₂(H₂O)−(SO₄)]. After few minutes at room temperature, cis-[PtCl₄{(15)NH₃}₂] precipitate was filtered, washed with cold water, and subsequently dried. This procedure yields the cis isomer with high purity, as confirmed by NMR (Figure 4) and infrared (IR) spectra (Figure S9 of Supporting Information).

LC–MS Instrumentation and Operating Conditions. All experiments were performed using an UltiMate 3000 UHPLC system coupled to a Velos Pro linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA). LC separation was performed at 30 °C on an Accucore Polar Premium C18 column (150 2.1 mm i.d., 2.6 mm particle size) equipped with an Accucore Polar Column (10 2.1 mm i.d.) security guard cartridge (Thermo Scientific, Waltham, MA, USA). Samples were injected into the column via a 5.0 μL sample loop. The following binary gradient elution program, containing 0.1% v/v of formic acid, was adopted: 0 min isocratic at 40% solvent B; 18 min linear from 40 to 100% (v/v) of solvent B; 12 min at 100% solvent B; 15–18 min back to the initial composition, followed by 10 min equilibration time. The flow rate was 200 μL/min.

MS full scan acquisitions were performed in positive ion mode, in the m/z range 150–2000. The following values were adopted for the Velos Pro heated ESI interface (Thermo Scientific) and ion optics parameters: sheath gas flow rate, 30 (arbitrary units); auxiliary gas flow rate, 15 (arbitrary units); spray voltage, 4 kV (negative polarity); capillary temperature, 275 °C; S-lens radio frequency level, 69 (arbitrary units). MS² (n = 2–3) acquisitions were performed on targeted precursor ions. For each fragmentation, an isolation window of 3 m/z centered on the m/z ratio of the most abundant isotope was used, in order to obtain also the isotopic pattern of product ions. Collision energy was typically 35% for both MS/MS (in this case a 400% value corresponds to a 100 V excitation voltage) and MS³. Control of LC–MS instrument and first processing of data were performed by the Xcalibur software 2.2 SP1.48 (Thermo Scientific). MS raw data were imported, further elaborated, and finally turned into figures by the Sigmaplot 12.5 software (Systat Software, Inc., London, UK). The ChemDraw Pro 8.0.3 software (CambridgeSoft Corporation, Cambridge, MA, USA) was employed to draw chemical structures. The m/z values listed in the text refer to the most intense peak of the isotopic clusters.

Preparation of Conjugates between CNCbl and Pt(II) Complexes. Stock solutions of cis-diamminedichloroplatinum(II) and hydrated cis-diammineplatinum(II)²⁰ 1.0 mg/mL were prepared in LC-grade water and sonicated for ca. 30 min. Adducts with CNCbl were synthesized as follows: 40 μL of stock solution of CNCbl (1 mg/mL in LC-grade water) was mixed in different vials with 125 μL of each of the platinum complexes. The resultant mixtures were kept under agitation at 60 °C for 16 h in 0.5 mL Eppendorf tubes. Each sample was pretreated with micro-C18 ZipTips columns (Millipore), using a mobile phase consisting of H₂O and CH₃OH 50:50 v/v containing both 0.1% HCOOH. The product of this purification was diluted 1:2 in a 0.1% HCOOH aqueous solution and then analyzed.

Solution NMR Experiments. (15)N-labeled CP (cis-[PtCl₄{(15)NH₃}₂]) was dissolved immediately prior to use in pure deoxygenated water. The complex solution was extensively vortexed and sonicated, and the exact Pt concentration was determined by atomic absorption spectrophotometry using a Varian 880Z instrument. (15)N-labeled CP was added to CNCbl and the reaction was monitored through 2D ¹H−²⁷N HSQC spectra, acquired in water with 10% D₂O (for the field-frequency lock) at 37 °C, using a gradient-enhanced sequence in which coherence selection and water suppression were achieved by gradient pulses. Sixteen transients were acquired over an F2 (¹H) spectral width of 14 ppm centered at 4.7 ppm, into 2048 data points for each of 128 t1 increments with an F1 (²⁷N) spectral width of 100 ppm centered at −50 ppm, using a delay 1/(4J_mn) of 2.78 ms and a recycle delay of 2.5 s. NMR spectra were collected on a Bruker AVANCE III 700 UltraShield Plus magnet, equipped with quadrupole resonance (QCI) CryoProbe, and processed using the standard Bruker software (TOPSPIN).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01879.

Scheme S1. Possible pathways leading to the generation of product ions detected at m/z 664.76 and m/z 656.10, from precursor ions at m/z 810 (811) and m/z 792 (793), respectively. Figure S1. ESI–MS spectrum obtained for CNCbl upon RPLC–MS analysis in a water/methanol mobile phase containing 25 mM formic acid. Figure S2. ESI–MS spectra of adducts formed between CNCbl and (15)N-labeled CP at m/z 811.28 (a) and (15)N-labeled cis-diammineplatinum(II) at m/z 793.29 (b). Figure S3. Suggested chemical structures of doubly charged conjugates formed by CNCbl with CP (left) and cis-diamminePt(II). Figure S4. Suggested structure of the protonated form of the NL of CNCbl, detected as product ion in the MS/MS spectra of both
investigated conjugates. Figure S5. Suggested fragmentation pathway to explain the generation of the doubly charged product ion at \( m/z \) 635.72 from the conjugate formed by CNCbl and cis-diammine-Pt(II). Figure S6. Suggested structure for the singly charged product ion at \( m/z \) 911.44 arising from the conjugate formed by CNCbl and the cis-diammine-Pt(II). Figure S7. Structure hypothesized to explain the product ion at \( m/z \) 616.01 detected in the MS\(^3\) spectrum related to the \( m/z \) 764.24 ion (see Scheme 1). Paragraph on multistage MS on conjugates formed by CNCbl and Pt(II) complexes. Table S1. Summary of product ions observed in CID–MS\(^3\) spectra. Figure S8. CID–MS\(^3\) spectra. Figure S9. IR spectra of cis-[Pt\(^{15}\text{NH}_3\)]\(_2\)(H\(_2\)O)\(\cdot\)\((\text{SO}_4)\) (a) and cis-[PtCl\(_2\)(\(^{15}\text{NH}_3)\)] (b) (PDF)

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### Notes

The authors declare no competing financial interest.

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## REFERENCES

(1) Kelland, L. The Resurgence of Platinum-Based Cancer Chemotherapy. *Nat. Rev. Cancer* 2007, 7, 573–584.

(2) Siddik, Z. H. Cisplatin: Mode of Cytotoxic Action and Molecular Basis of Resistance. *Oncogene* 2003, 22, 7265–7279.

(3) Dusari, S.; Tchounoum, P. B. Cisplatin in Cancer Therapy: Molecular Mechanisms of Action. *Eur. J. Pharmocol.* 2014, 7, 364–378.

(4) Johnstone, T. C.; Suntharalingam, K.; Lippard, S. J. The Next Generation of Platinum Drugs: Targeted Pt(II) Agents, Nanoparticle Delivery, and Pt(IV) Prodrugs. *Chem. Rev.* 2016, 116, 3436–3486.

(5) Wang, D.; Lippard, S. J. Cellular Processing of Platinum Anticancer Drugs. *Nat. Rev. Drug Discovery* 2005, 4, 307–320.

(6) Collins, D. A.; Hogenkamp, H. P. C.; O’Connor, M. K.; Naylor, S.; Benson, L. M.; Hardymon, T. J.; Thorson, L. M. Biodistribution of Radiolabeled Adenosylcobalamin in Patients Diagnosed with Various Malignancies. *Mayo Clin. Proc.* 2000, 75, 568–580.

(7) García-Sar, D.; Montes-Bayón, M.; Blanco González, E.; Sanz-Medel, A. Speciation Studies of Cisplatin Adducts with DNA Nucleotides via Elemental Specific Detection (P and Pt) Using Liquid Chromatography–Inductively Coupled Plasma-Mass Spectrometry and Structural Characterization by Electrospray Mass Spectrometry. *J. Anal. At. Spectrom.* 2006, 21, 861–868.

(8) García Sar, D.; Montes-Bayón, M.; Aguado Ortiz, L.; Blanco-González, E.; Sierra, L. M.; Sanz-Medel, A. In Vivo Detection of DNA Adducts Induced by Cisplatin Using Capillary HPLC–ICP-MS and Their Correlation with Genotoxic Damage in *Drosophila Melanogaster*. *Anal. Bioanal. Chem.* 2008, 390, 37–44.

(9) Ventura, G.; Arnesano, F.; Calvano, C. D.; Palmisano, F.; Cataldi, T. R. I. Cyanocobalamin conjugates of cisplatin and diaminocyclohexane-platinum(ii): matrix-assisted laser desorption ionization mass spectrometry characterization using 4-chloro-α-cyanocinnamic acid as the matrix. *RSC Adv.* 2017, 7, 53658–53666.

(10) Güssöcher, S.; Gruber, K.; Kratky, C.; Eichmüller, C.; Kräutler, B. B12-retro-Riboswitches: Constitutional Switching of B12 Coenzymes Induced by Nucleotides. *Angew. Chem., Int. Ed.* 2005, 44, 2284–2288.

(11) Kräutler, B. B12-Coenzymes, the Central Theme. In *Vitamin B12 and B12-Proteins* 2nd ed.; Kräutler, B., Arigoni, D., Golding, B. T., Eds.; Wiley-VCH Verlag GmbH: Weinheim, Germany, 2007; pp 3–43.

(12) Mundwiler, S.; Spangler, B.; Kurz, P.; Kunze, S.; Alberto, R. Cyanide-Bridged Vitamin B12–Cisplatin Conjugates. *Chem.—Eur. J.* 2005, 11, 4089–4095.

(13) Ruiz-Sánchez, P.; Mundwiler, S.; Spangler, B.; Buan, N. R.; Escalante-Semerena, J. C.; Alberto, R. Syntheses and Characterization of Vitamin B12–Pt(II) Conjugates and Their Adenosylation in an Enzymatic Assay. *J. Biol. Inorg Chem.* 2008, 13, 335–347.

(14) Ruiz-Sánchez, P.; König, C.; Ferrari, S.; Alberto, R. Vitamin B12 as a Carrier for Targeted Platinum Delivery: In Vitro Cytotoxicity and Mechanistic Studies. *J. Biol. Inorg Chem.* 2011, 16, 33–44.

(15) Messori, L.; Merlini, A. Cisplatin Binding to Proteins: A Structural Perspective. *Coord. Chem. Rev.* 2016, 315, 67–89.

(16) Hostetter, A. A.; Osborn, M. F.; DeRose, V. J. RNA-Pt Adducts Following Cisplatin Treatment of *Saccharomyces Cerevisiae*. *ACS Chem. Biol.* 2012, 7, 218–225.

(17) Speelmans, G.; Staffhorst, R. W. H. M.; Versluis, K.; Reedijk, J.; de Kruiff, B. Cisplatin Complexes with Phosphatidylserine in Membranes. *Biochemistry* 1997, 36, 10545–10550.

(18) Tran, M. T. Q.; Stūrup, S.; Lambert, I. H.; Gammelgaard, B.; Furger, E.; Alberto, R. Cellular Uptake of Metallated Cobalamins. *Metallomics* 2016, 8, 298–304.

(19) Vinje, J.; Sletten, E. NMR Spectroscopy of Anticancer Platinum Drugs. *Adv. Anticancer Agents Med. Chem.* 2007, 7, 35–54.

(20) Jaliléhová, F.; Laffin, L. J. Structure of the Hydrated Platinum(II) Ion and the cis-Diammineplatinum(II) Complex in Acidic Aqueous Solution: An EXAFS Study. *Inorg. Chem.* 2008, 47, 3248–3254.

(21) Calvano, C. D.; Ventura, G.; Palmisano, F.; Cataldi, T. R. I. 4-Chloro-α-cyanocinnamic acid is an efficient soft matrix for cyanocobalamin detection in foodstuffs by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). *J. Mass Spectrom.* 2016, 51, 841–848.

(22) Mejía, J.; Coplen, T. B.; Berglund, M.; Brand, W. A.; De Bievre, P.; Grönig, M.; Holden, N. E.; Irreger, J.; Loss, R. D.; Walczyk, T.; et al. Isotopic Compositions of the Elements 2013 (IUPAC Technical Report). *Pure Appl. Chem.* 2016, 88, 293–306.

(23) Waibel, R.; Treichler, H.; Schaefer, N. G.; van Staveren, D. R.; Mundwiler, S.; Kunze, S.; Ku, M.; Alberto, R.; Nu, J. New Derivatives of Vitamin B12: Show Preferential Targeting of Tumors. *Cancer Res.* 2008, 68, 2904–2911.

(24) Zelder, F.; Alberto, R.; Kadish, K. M.; Smith, K. M.; Guillard, R. Vitamin B12 Derivatives for Spectroanalytical and Medicinal Applications. *Handbook of Porphyrin Science*; World Scientific, 2012; Vol. 25, pp 83–130.

(25) Berners-Price, S. J.; Ronconi, L.; Sadler, P. J. Insights into the Mechanism of Action of Platinum Anticancer Drugs from Multi-nuclear NMR Spectroscopy. *Prog. Nucl. Magn. Reson. Spectros.* 2006, 49, 65–98.

(26) Dhara, S. C. Cisplatin. *Indian J. Chem.* 1970, 8, 123–134.

(27) Rochon, F. D.; Melanson, R. Molecular and Crystal Structure of a Platinum(II) Complex with Aqua and Sulfate Ligands: *Aqua(N,N‘-Dimethylthelyleneediamine)(Sulfato)Platinum(II)* Hydrate. *Inorg. Chem.* 1987, 26, 989–992.

(28) Appleton, T. G.; Hall, J. R.; Ralph, S. F.; Thompson, C. S. M. Reactions of Platinum(II) Aqua Complexes. 2. *Pt NMR Study of Reactions between the Tetraaquaplatinum(II) Cation and Chloride, Hydroxide, Perchlorate, Nitrate, Sulfate, Phosphate, and Acetate.* *Inorg. Chem.* 1994, 23, 3521–3525.