The protein content of the solubilized receptor was determined,[22] and it was then divided into 1-mL fractions, which were rapidly frozen in liquid nitrogen and stored at −70 °C until needed. Inhibition experiments were performed under competitive conditions where 50 μg of soluble receptor protein (10 μL) was added to 1 mL of assay medium containing 5 nM [^{3}H]ryanodine (60 Ci/mmol), a desired concentration of test ryanoid, 0.5 M NaCl, 0.1% CHAPS, 300 μM $CaCl_2$, and 40 mM Tris/maleate, pH 7.1. Samples were allowed to equilibrate for 80 min at 37 °C followed by rapid filtration through Whatman GF/C glass fiber filters.[10,11]

Specific binding (>95% in each case) was defined as the difference between total binding (with [^{3}H]ryanodine alone) and nonspecific binding (with [^{3}H]ryanodine fortified with 10 mM unlabeled ryanodine). Concentrations for 50% inhibition (IC_{50} values) were determined from regression analyses of Hill data.[23] The 95% confidence limits had an average range of ±3% of the IC_{50} values.

Toxicity to Mice. LD_{50} values were determined from mortality data 24 h after ip administration of the ryanoid to male albino Swiss-Webster mice (18–22 g) with 50% aqueous ethanol or methoxytriglycol (10–100 μL) as the carrier vehicle compared with appropriate controls. The reported LD_{50}'s are based on plots of logarithmic dose vs. probit percent mortality. They are approximations, due to the small amounts of test compounds, but are estimated to fall within 1.5-fold of the actual values.

Toxicity to Insects. The standard knockdown (KD) assay used adult female houseflies (*Musca domestica*, SCR susceptible strain) treated by intrathoracic injection of a 50% aqueous ethanol solution (1 μL) of the test compound 2 h after topical treatment on the abdomen with 7.5 μg of PB applied in 0.5 μL of acetone. The KD endpoint was the number of flies immobilized 4 h posttreatment, the KD_{50} being determined from logarithmic dose–probit KD plots. In other studies, houseflies (some pretreated topically with 7.5 μg of PB and others not) were administered a ryanoid topically or by injection and adult male American cockroaches (*Periplaneta americana*) were treated by intracoxal injection with 1 or 17 in 50% aqueous ethanol (10 μL) as above. KD_{50} and LD_{50} values are based on two independent experiments with a dose differential of 1.5–2-fold and 10 houseflies or six cockroaches for each dose.

Metabolism of [^{3}H]Ryanodine in Houseflies. Ten adult female houseflies were injected as above with [^{3}H]ryanodine (1.1×10^6 dpm, 160 μg/g). Each batch of treated flies was held in a 100-mL glass beaker for 1 or 4 h, at which time the flies were removed and the flies and their excreta were separately extracted with MeOH, with tritium recovery values of 55%, 21%, and 4% at 1 h and 38%, 40%, and 6% at 4 h, for the flies, excreta, and unextracted residue, respectively. Methanol extracts of the 1-h excreta and the flies 4 h after treatment were subjected to TLC on 0.20-mm silica gel plates (15% MeOH/$CHCl_3$) with metabolite detection by liquid scintillation counting. These TLC conditions gave R_f values of 0.46 and 0.00 for 1 and 17, respectively. A comparable excreta extract was subjected to analytical HPLC [0% MeOH (1 min), 0–100% MeOH (10 min); R_T = 12.5 min (1), 10.5 min (17)], comparing the standard compounds and the ^{3}H-labeled metabolites.

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Comparative Antitumor Studies on Platinum(II) and Platinum(IV) Complexes Containing 1,2-Diaminocyclohexane

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The synthesis and characterization of a group of platinum(IV) compounds containing the various isomeric forms of 1,2-diaminocyclohexane (DACH) are described. Antitumor tests with the new complexes, as well as with other platinum(II) compounds containing the DACH ligand, revealed that *trans,cis*-Pt^{IV}(*SS*-DACH)$(OH)_2Cl_2$, **7**, is more active than its mirror image, *trans,cis*-Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, **6**, against L1210 leukemia implanted in mice. However, activity is dependent on the tumor model, and against B16 melanoma implanted in mice, the activities of the two enantiomers are reversed, with **6** being more active than **7**. The results of the tests are discussed in light of the mechanism by which Pt(IV) compounds are believed to express their antitumor effects.

Since the first report of the anticancer activity of *cis*-dichlorodiammineplatinum(II), **1**, by Rosenberg et al.,[1] a wide variety of platinum compounds have been synthesized and examined for their antitumor activity.[2] Extensive study of the structure–activity relationships of the platinum-based anticancer agents has revealed that the most active compounds are those having cis-coordination sites occupied by mono- or bidentate amine ligands (or ammonia) and two sites occupied by weak trans-directing groups, e.g., Cl^-, NO_3^-, SO_4^{2-}, etc. On the molecular level, the compounds are believed to express their antitumor effects by loss of the weak trans-directing groups and subsequent binding of the platinum nucleus to DNA.[3-5]

In addition to antitumor active Pt(II) complexes, oxidation of a variety of divalent compounds with H_2O_2, Cl_2, or Br_2 yields 6-coordinate complexes of Pt(IV), which are themselves generally active as anticancer agents.[1,6-10] In light of the fact that substitution reactions on Pt(IV) are

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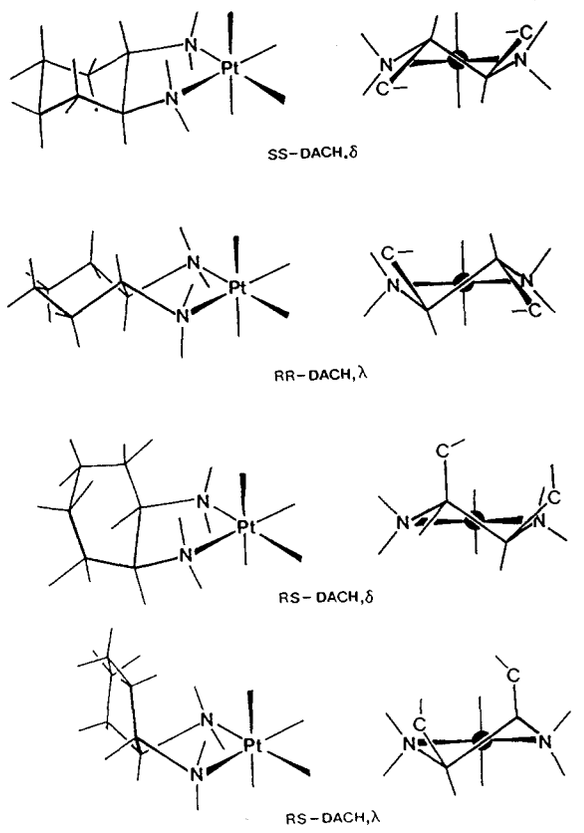

Figure 1. The stereochemistry of the various isomeric forms of coordinated 1,2-diaminocyclohexane, DACH, is shown.

slow, relative to those on Pt(II),[11] it has been suggested that Pt(IV) complexes such as *cis,cis,trans*-$Pt^{IV}(NH_2CH(CH_3)_2)_2Cl_2(OH)_2$, **2**, express their antitumor effects via in vivo reduction to biologically active platinum(II) compounds.[10,12-17] In support of this suggestion is the observation that significant amounts of Pt(II) compounds have been found in the urine and blood of cancer patients receiving **2**.[14] In addition, Van der Veer et al.[17] have recently reported that reaction of the Pt(IV) complex **2** with 5′-guanosine monophosphate results in a Pt(II) product that possesses the two isopropylamine ligands as well as two coordinated mononucleotides. Thus, under certain conditions, DNA itself or its components may act as reducing agents for Pt(IV). Interestingly, incubation of purified calf thymus DNA with **2** for extended periods of time (weeks) has been reported to result in DNA platination.[18] However, neither the oxidation sate of the metal ion bound to DNA nor the exact nature of the platinum–DNA adduct were established in the latter study.

The antitumor properties of platinum(II) complexes containing optically active amine ligands have been investigated by a number of researchers.[19-26] The most actively studied complexes are those containing the isomeric forms of 1,2-diaminocyclohexane (DACH), Figure 1. Although numerous Pt(II) complexes of DACH have been examined for activity against Sarcoma 180 and the leukemias P388 and L1210, the antitumor activity of platinum(IV) compounds containing the isomerically pure forms of the diamine have not been reported. In an effort to examine if, and to what extent, the presence of optical activity in the amine portion of a platinum(IV) complex influences antitumor activity, we have conducted a comparative study of a group of platinum(II) and platinum(IV) compounds containing the isomerically pure forms of DACH. In this paper we describe the synthesis of the compounds and present their antitumor activity against leukemia L1210 and B16 melanoma.

Experimental Section

Infrared spectra (4000–250 cm^{-1}) of Nujol mulls of the compounds between KBr disks were obtained on a Beckman 4220 IR spectrometer. The ^{195}Pt NMR spectral data (at 77.25 MHz) were collected in dimethylformamide solution (~40 mM) at 25 °C, on a Bruker WM-360 wide-bore NMR spectrometer. The chemical shift data are reported relative to external K_2PtCl_6 dissolved in D_2O. Chemical analyses were carried out by the Bristol-Myers Co.

Antitumor Activity. The specific methods for the antitumor testing have been reported in detail elsewhere.[2,8] The L1210 experiments were performed in CDF_1 mice implanted ip with 1×10^6 L1210 cells. The complexes, suspended in 0.9% NaCl solution with a drop of Tween 80, were administered ip on day 1 after tumor inoculation. Drug-treated groups consisted of five mice, with a leukemic control group of 10 mice for each study. The B16 experiments were initiated by implanting BDF_1 mice ip with 0.5 mL of a 10% tumor brei suspension. The complexes were administered ip daily for 9 days, beginning on day 1 after implant. Drug-treated and control groups consisted of 10 mice. Groups treated with *cis*-$Pt^{II}(NH_3)_2Cl_2$, 1, were included in each L1210 and B16 study for comparison.

With both experimental tumor models, mice were observed daily and antitumor activity was determined on the basis of increases in lifespan of treated mice relative to untreated controls. Increases in lifespan were expressed as % T/C, defined as the median survival time (MST) in a drug-treated group (T) divided by the MST of the untreated control group, times 100.

Pt^{II}(*RR*-DACH)Cl_2, 3. A solution of 1.50 g (3.62 mmol) of potassium tetrachloroplatinate in 15 mL of distilled water was filtered to remove any undissolved substances. The solution was placed in a 50-mL round-bottom flask, and 0.413 g (3.62 mmol) of *RR*-DACH dissolved in 20 mL of water was added dropwise with stirring. After 1 h, the bright yellow solid that formed was removed by filtration, washed with water, acetone, and ether, and air-dried. The yield was 1.05 g, 73%. Anal. ($H_{14}Cl_2C_6N_2Pt$) H, Cl, C, N, Pt.

Pt^{II}(*SS*-DACH)Cl_2, 4. Working on a scale of 0.80 g (7.07 mmol) of *SS*-DACH, the compound was synthesized in a manner identical with that described for **3**. The yield was 1.5 g or 56%.

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Table I. Infrared and ^{195}Pt NMR Data for the Compounds

compound[a]	IR, cm^{-1} OH	NH	Pt—Cl	C=O	^{195}Pt NMR,[b] δ
Pt^{II}(*RR*-DACH)Cl_2, **3**		3280 s	305 m		−2287 (380)
Pt^{II}(*SS*-DACH)Cl_2, **4**		3192 s			
		3102 s			
Pt^{II}(*RS*-DACH)Cl_2, **5**		3250 s	325 m		−2284 (300)
		3195 s			
		3125 s			
trans,cis-Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, **6**	3490 s	3200 s	310 m		
trans,cis-Pt^{IV}(*SS*-DACH)$(OH)_2Cl_2$, **7**		3100 s			
trans,cis-Pt^{IV}(*RS*-DACH)$(OH)_2Cl_2$, **8**	3485 s	3225 s	320 m		
		3445 s			
Pt^{II}(*RR*-DACH)(ox), **9**		3210		1705 s	−1995 (640)
Pt^{II}(*SS*-DACH)(ox), **10**		3180 s		1665 s	
		3090 s			

[a] Abbreviations: DACH = 1,2-diaminocyclohexane, *RR*-DACH = trans-*l*, *SS*-DACH = trans-*d*, *RS*-DACH = cis, ox = the oxalate anion. [b] NMR data were collected in dimethylformamide solution and referenced to external Na_2PtCl_6 in D_2O. The line width, in Hz, is given in parentheses. Due to limited solubility, the ^{195}Pt NMR data for **6–8** were not obtained.

Anal. ($H_{14}C_6Cl_2N_2Pt$) H, Cl, C, N, Pt.

Pt^{II}(*RS*-DACH)Cl_2, 5. A solution of 2.00 g (4.82 mmol) of K_2PtCl_4 in 25 mL of water was filtered to remove any undissolved substances. Nine-tenths of a gram (4.82 mmol) of *RS*-DACH·2HCl[27] was dissolved in 10 mL of water and the pH of the resulting solution adjusted to 10.5 by addition of 1 N aqueous NaOH. The basic amine solution was added dropwise to the solution containing K_2PtCl_4 with stirring. After 90 min, the pale yellow precipitate that formed was removed by filtration, washed with water, acetone, and ether, and air-dried. The yield was 1.32 g or 72%. Anal. ($H_{14}Cl_2C_6N_2Pt$) H, Cl, C, N, Pt.

***trans,cis*-Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, 6.** To a stirred suspension of 0.302 g (0.79 mmol) of **3** in 10 mL of distilled water was added dropwise 12 mL of 30% aqueous H_2O_2. After the mixture was stirred for 1 h, the bright yellow solid, **3**, changed to a very pale yellow color. The pale yellow solid was removed by filtration, washed with water, acetone, and ether, and air-dried. The yield was quantitative. Anal. ($H_{16}C_6Cl_2N_2O_2Pt$) H, Cl, C, N, Pt.

***trans,cis*-Pt^{IV}(*SS*-DACH)$(OH)_2Cl_2$, 7.** With 0.70 g (1.84 mmol) of **4** as starting material, this compound was synthesized in a manner identical with that described for **6**. The yield was quantitative. Anal. ($H_{16}Cl_2C_6N_2O_2Pt$) H, Cl, C, N, Pt.

***trans,cis*-Pt^{IV}(*RS*-DACH)$(OH)_2Cl_2$, 8.** To a stirred suspension of **5** (0.836 g, 2.20 mmol) in 15 mL of water was added dropwise 10 mL of 30% aqueous H_2O_2. The reaction was allowed to proceed at room temperature for 1 h. The pale yellow precipitate that formed was removed by filtration, washed with water, acetone, and ether, and air-dried. The yield was quantitative. Anal. ($H_{16}Cl_2C_6N_2O_2Pt$) H, Cl, C, N, Pt.

Pt^{II}(ox)(*RR*-DACH), 9, and Pt^{II}(ox)(*SS*-DACH), 10. These compounds were made in the manner reported by Kidani et al.[23]

Results and Discussion

The Metal Complexes. The binding of the various isomeric forms of 1,2-diaminocyclohexane to either Pt(II) or Pt(IV) confers specific conformations to the bound ligand. For the enantiomeric pair *RR*- and *SS*-DACH, binding of both amino groups of the diamine to the metal ion results in five-membered chelate rings that are not flat but possess the λ (*RR*) and δ (*SS*) conformations, Figure 1. Moreover, while these chelate-ring conformations are similar to those found for coordinated 1,2-diaminoethane, the presence of the cyclohexane ring in DACH does not allow facile interconversion between the λ and δ forms as is the case for the former ligand. Thus, complexation yields rigid structures possessing a cyclohexane chair conformation of the type found in the complexes Pt^{II}(malonato)(*RR*-DACH)[28] and [Co(*SS*-DACH)$_3$]Cl_3·H_2O.[29] It is this ligand conformation that is present in the 4- and 6-coordinate complexes **3**, **4**, **6**, **7**, **9**, and **10** employed in this study.

Crystallographic[30] and ^{13}C NMR studies [23] indicate that the coordinated form of the meso ligand, *RS*-DACH, is significantly different than its coordinated optically active forms. The five-membered chelate ring can adopt λ or δ conformations, which in this case easily interconvert. Perhaps more importantly, the absolute configurations of the two asymmetric centers in the ligand constrains the cyclohexane ring to lie out of the plane defined by the two nitrogen donor atoms and the platinum ion, Figure 1. Thus, for square-planar compounds such as Pt^{II}(*RS*-DACH)Cl_2, **5**, considerable steric bulk lies out of the N_2Cl_2 donor plane of the complex. This structural property sharply contrasts with that of the enantiomeric pair **3** and **4** wherein the cyclohexane moiety is constrained to lie mainly near the N_2Cl_2 donor plane. Similar arguments pertaining to the bound DACH ligand apply to the 6-coordinate compounds **6–8**.

Inspection of the data given in Table I reveals that *trans,cis*-Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, **6**, and its mirror image **7**, which contain symmetry-equivalent OH groups, give rise to a single OH stretching mode at 3490 cm^{-1}. However, this is not the case for **8**, which due to the presence of the cyclohexane ring on one side of the nitrogen–metal–nitrogen plane possesses nonequivalent OH groups. As expected, this isomer yields two OH stretching modes at 3485 and 3445 cm^{-1}. Infrared differences between the optically active and the meso forms of the compounds are also apparent in the locations of the Pt–Cl stretching modes ~300 cm^{-1}, Table I.

The ^{195}Pt NMR chemical shifts for compounds **3–5**, obtained in dimethylformamide solution, are consistent with a Pt(II) ion ligated by two nitrogen and two chloride donor ligands.[31] Although the disposition of the cyclohexane rings in the enantiomeric pair **3** and **4** is different than that of the ring in **5**, the difference is not manifested in the position of the NMR resonance. Within experimental error, all three compounds exhibit the same ^{195}Pt NMR chemical shift in DMF solution. As expected, substitution of two chloride ions of **3** and **4** with the more strongly trans-labilizing ligand oxalate significantly deshields the platinum ion (Table I, compounds **9** and **10**,

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Table II. Optimal Effects and Doses of Pt Analogues against L1210 and B16[a]

analogue	L1210 maximum % T/C	L1210 optimal dose,[b] day 1	B16 maximum % T/C	B16 optimal dose, qd 1–9
1[c]	150–207	6–10	167–229	1.6–2
3, Pt(II)(*RR*)	242	8	160	1.6
4, Pt(II)(*SS*)	229	16	162	1.6
5, Pt(II)(*RS*)	200[d]	10	*f*	
	163[e]	6		
6, Pt(IV)(*RR*)	158[d]	6	200[d]	2.4
	171[e]	12	203[e]	3.6
7, Pt(IV)(*SS*)	257[d]	128	160[d]	8
	243[e]	64	162[e]	6.4
8, Pt(IV)(*RS*)	143[d]	40	*f*	
	129[e]	80		
9, Pt(II)(*RR*)	179	10	178	1.6
10, Pt(II)(*SS*)	143	10	178	2.4

[a] Mice were implanted ip with 10^6 L1210 ascites cells or 0.5 mL of a 10% B16 tumor brei suspension. Treatment groups consisted of five mice (L1210 experiments) or 10 mice (B16); the treatment route was ip. [b] The dose, in milligrams/kilogram per injection, producing the maximum % T/C without severe toxicity (deaths > 17%). [c] *cis*-Diamminedichloroplatinum(II). [d] Run 1. [e] Run 2. [f] Not tested.

causing the ^{195}Pt NMR resonance to occur ~300 ppm to lower field relative to the resonances of the former compounds.

Antitumor Data and Mechanism. The results of the antitumor testing from individual experiments are summarized in Table II. All eight of the compounds tested against L1210 were active (% T/C ≥ 125), and some, e.g., Pt^{II}(*RR*-DACH)Cl_2, **3**, and Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, **6**, had activity above the range obtained for *cis*-$Pt^{II}(NH_3)Cl_2$, **1**. Comparison among the Pt(II) complexes containing the various isomeric forms of the DACH ligand revealed that the compounds with the *RR* form tended to be more active than those with either the *SS* or meso forms. This finding, which agrees with earlier studies with the Pt(II) compounds,[20,21] may be due to the different abilities of the various complexes to platinate DNA.[26] Table II also shows that the ability of ligand stereochemistry to influence antitumor activity is dependent on the tumor model and no activity differences between the enantiomeric pairs **3**,**4** and **9**,**10** vs. B16 were found. Although the difference in treatment schedule used for L1210 and B16 may in part be responsible for this observation, a similar lack of differentiation between these enantiomeric Pt(II) compounds has been noted for both P388[19,20] and Sarcoma 180.[23] The potencies of all of the Pt(II) compounds studied, Table II, were comparable to that of **1** in either tumor model.

A comparison of the results obtained with the Pt(IV) enantiomeric pair of complexes *trans,cis*-Pt^{IV}(*RR*-DACH)$(OH)_2Cl_2$, **6**, and *trans,cis*-Pt^{IV}(*SS*-DACH)-$(OH)_2Cl_2$, **7**, is worthy of note. Although the initial L1210 studies, Table II, run 1, were not done concurrently, the results suggested that **7** was more active and less potent than **6**. These two complexes were tested again vs. L1210 in a heads-on comparison, run 2, Table II, and again, **7** was more active and less potent than **6**. The compounds were tested concurrently twice against B16, and the results showed that against this tumor **6** was more active than **7**. The *RR* form was also somewhat more potent, but by a lesser margin than against L1210. Thus, while the results against L1210 showed the *SS* form to be the more active of the mirror image pairs, the activity of the two enantiomers was reversed against B16. The complex containing the meso form of the ligand, **8**, proved to be the least active of the Pt(IV) compounds tested, Table II.

Although interpretation of antitumor activity data in terms of the specific chemical and biochemical events that underlie the cytotoxic properties of antitumor compounds is difficult, some general observations pertaining to the probable mechanism of action of the compounds employed in this study are possible. On the molecular level, Pt(IV) antitumor agents are believed at least in part to exert their cytotoxic effects by reduction to active Pt(II) compounds.[10,12-17] Although the reduction behavior of compounds **6**–**8** has not been investigated, the complexes should have reduction potentials similar to the Pt(IV) antitumor agents *cis,cis,trans*-$Pt^{IV}(NH_2CH(CH_3)_2)_2Cl_2(OH)_2$, **2**, and *cis,cis,trans*-$Pt^{IV}(NH_3)_2Cl_2(OH)_2$. Furthermore, reduction of **6**–**8** would be expected to yield the Pt(II) compounds **3**–**5**, respectively.[12] Since Pt(II) complexes easily undergo substitution reactions, the Pt(II) products would be expected to react readily with cellular components, e.g., DNA. Recent studies with **3**–**5** have shown that the compounds can platinate DNA and that the type of DNA adduct that is formed is sensitive to ligand stereochemistry.[26,32] Inspection of the antitumor data in Table II reveals variances both in potency and antitumor effects between Pt(II) and Pt(IV) complexes. The role of the rate of reduction and pharmacologic properties in affecting these differences remains to be determined.

A second observation concerns the reversal in the relative activities of **6** and **7** when the tumor model is altered from L1210 to B16. This result was unexpected, and it demonstrates that at least for these enantiomeric Pt(IV) compounds tumor model and/or the method of testing can be important factors in determining compound activity. Considering the differences in treatment schedule, single dose for L1210 and nine doses for B16, the results suggest that **7** may have schedule dependency with possibly increased toxicity with daily treatment.

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