Journal of Medicinal Chemistry

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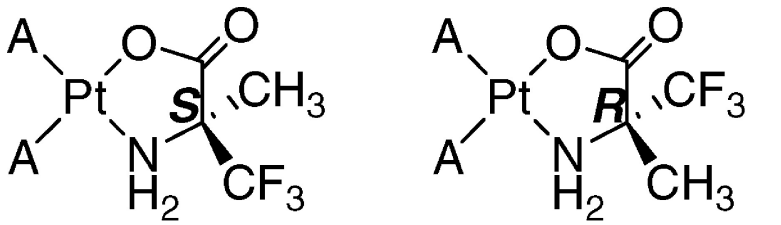
J. Med. Chem., **2005**, 48 (24), 7821-7828• DOI: 10.1021/jm0504003 • Publication Date (Web): 01 November 2005



$A_2 = Cl_2$, $(NH_3)_2$, en, phen, Me_2phen

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Platinum-Based Antitumor Drugs Containing Enantiomerically Pure α-Trifluoromethyl Alanine as Ligand

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Received April 26, 2005

Synthetic amino acids such as fluorinated α-amino acids are currently actively investigated for their biological activity. Herein, we report on the synthesis and characterization of platinum complexes containing an N,O-chelated pure enantiomer of α-trifluoromethylalanine (α-Tfm-Ala). The compounds are either anionic, $K[PtCl_2(\alpha\text{-Tfm-Ala})]$, or cationic, $[PtAm_2(\alpha\text{-Tfm-Ala})](NO_3)$ ($Am_2 = (NH_3)_2$, ethylendiamine (en), 1,10-phenanthroline (phen), and 2,9-dimethyl-1,10-phenanthroline (Me_2phen)). All complexes are highly soluble in water and have been fully characterized by NMR spectroscopy. In vitro cytotoxicity assays on different human tumor cell lines have been performed on some of the isolated compounds. $[Pt(NH_3)_2(\alpha\text{-Tfm-Ala})]$ with *R* configuration of the amino acid proved to have an activity comparable to that of the reference compound cisplatin. Flow cytometric analysis on NCI-H460 tumor cells (absence of G_2/M arrest, which instead is observed in the case of cisplatin) suggests a mechanism of action different from that of cisplatin.

Introduction

Complexes of platinum with amino acids, peptides, and proteins have been studied for a long time, particularly after Rosenberg's discovery of the antitumoral activity of platinum compounds.[1−3] Amino acids are essential starting materials for cell growth, being building blocks for protein synthesis; therefore, platinum complexes with amino acids could have a preferential affinity for cancerous cells subject to accelerated metabolism, duplication, and growth speed.[4−7]

Amino acids can also improve the cell uptake of antitumoral agents, since cellular membranes contain specific carriers for amino acids. Finally, it has been shown that platinum amino acid complexes can react with DNA, releasing the amino acidic ligand and forming the classical Pt−DNA cross-links that are responsible for cellular damage and, consequently, antitumor activity of platinum complexes.[8] The use of platinum(II) species as a protecting group in peptide synthesis has also been investigated.[9]

Amino acids can coordinate to platinum in different ways. They can be monodentate (coordinated only through the amino group or only through the carboxylic functionality)[10] or bidentate (coordinated through both the amino and the carboxylic groups).[11−14] If the amino acid contains a sulfur atom in a side chain, there is also the possibility of S coordination.[15] In this context it is worth mentioning that sulfur-containing amino acids have been extensively studied as models for platinum interaction with glutathione (GSH) and metallothioneines. These latter interactions are believed to play a role in the side effects of platinum drugs, especially at the kidney level.[16,17]

Notwithstanding the huge number of platinum complexes with amino acids that have been synthesized and tested, none of them appear to have reached the stage of clinical trials, indicating that natural amino acids, as ligands, are probably not suitable either for conferring to platinum compounds activity toward other types of tumors or for reducing the drawbacks of platinum drugs in current clinical use.[2,18−23]

More recently, the attention has been directed toward synthetic amino acids. This interest stems from the relevant biological activity shown by some of them and from the fact that peptides incorporating non-natural amino acids have shown an increased metabolic stability and, in some cases, also enhanced biological activity.[24,25] In particular, α-trifluoromethyl-α-amino acids (α-Tfm-AA) have proven to be extremely interesting analogues of natural α-amino acids, owing to some unique properties of the xenobiotic CF_3 group, such as high electron density, low toxicity, moderate steric hindrance, and hydrophobic character.[26,27] For example, peptidomimetic drugs having potential antithrombotic activity have been prepared by incorporating α-Tfm-arginine or α-Tfm-aspartic acid in RGD (Arg-Gly-Asp) sequences.[28] As a matter of fact, one of the fundamental events in the thrombus formation is the binding of fibrinogen to its receptors mediated by the amino acidic sequence RGD. Therefore, since peptides mimicking the fibrinogen receptor binding domain could be used in antithrombotic therapy, several compounds containing the RGD sequence or a variant of this tripeptide have been syn-

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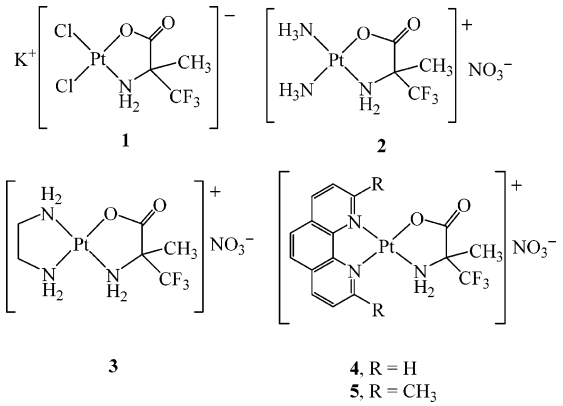
10.1021/jm0504003 CCC: $30.25
Published on Web 11/01/2005

Scheme 1. Sketches of the Novel Compounds[a]

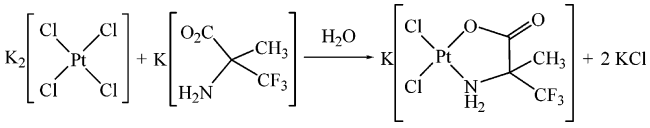


1 **2**

3 **4**, R = H; **5**, R = CH_3

[a] The chirality of the aminoacid is not indicated.

Scheme 2. Reaction Scheme for the Synthesis of K[$PtCl_2$(α-Tfm-Ala)]



thesized and tested.[29] To improve the stability of the secondary structure of the peptide, fluorinated $C^{\alpha,\alpha}$-disubstituted amino acids have been inserted in key positions of peptidomimetics. Such a substitution, besides enhancing its resistance to proteolytic enzymes, is also likely to influence the ternary structure of the peptide and its lipophilicity.[30]

It should also be pointed out that the synthesis of non-naturally occurring optically pure chiral ligands is rather challenging. This is especially true for fluorinated amino acids whose asymmetric synthesis often requires complex experimental protocols and uneasy to handle starting materials.

The preparation in our laboratories of several optically pure fluorinated α-amino acids by means of an efficient and versatile synthetic protocol,[24,25,31,32] prompted us to investigate their behavior as ligands for platinum and to test the antitumor activity of the newly synthesized compounds.

In this paper we report on a series of platinum(II) complexes containing optically pure α-trifluoromethylalanine as ligand (Scheme 1). To the best of our knowledge this is the first reported synthesis and characterization of platinum(II) complexes containing an optically pure α-Tfm-AA.[33]

Results and Discussion

Synthesis and Characterization. The enantiomeric anionic complexes [$PtCl_2$(*S*-α-Tfm-Ala)]$^-$ and [$PtCl_2$(*R*-α-Tfm-Ala)]$^-$ (compounds **1*S*** and **1*R***, respectively) were prepared from $K_2[PtCl_4]$ and the pure enantiomers of α-Tfm-Ala as reported in Scheme 2. The corresponding potassium salts were very soluble in water and highly hygroscopic.

The peak at *m/z* 422 (with reference to the most abundant peak in the isotopic cluster), in the negative MALDI TOF spectrum of complex **1*R*** in water, is assigned to the parent ion M^- ($C_4H_5Cl_2F_3NO_2Pt$). The coordination of the amino acid to the metal is proven by the shift, in the ^1H NMR spectrum in D_2O, of the

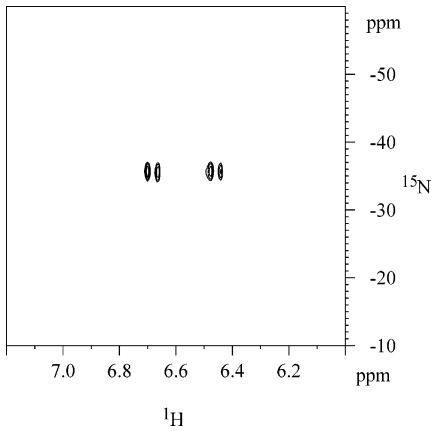
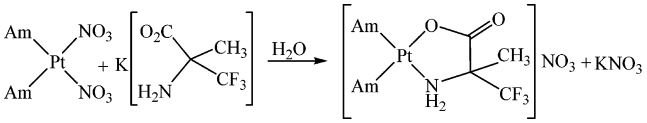


Figure 1. $^1H-^{15}N$ HSQC (H_2O/D_2O 90/10, v/v; pH 4.05) of [$PtCl_2$(*S*-α-Tfm-Ala)]$^-$.

Scheme 3. Reaction Scheme for the Synthesis of [$PtAm_2$(α-Tfm-ala)](NO_3)[a]



[a] Am_2 Stands for Two Ammines or a Chelating Diamine.

methyl group, which falls at 1.89 ppm in the complex and at 1.72 ppm in the free amino acid (note that the free amino acid is in a zwitterionic form; therefore, coordination to platinum corresponds to substitution of a Pt^{2+} for a H^+). Evidence in favor of the N,O-chelation of the amino acid comes from the chemical shift of ^{195}Pt, which is slightly shifted with respect to the signal of the starting platinum complex ($\delta = -1627$ ppm in **1** and -1615 ppm in $K_2[PtCl_4]$) and falls in a range that is typical of a Cl_2NO environment.

The 2D $^1H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectrum acquired in H_2O/D_2O (90:10 v/v, Figure 1) exhibits two cross-peaks with a doublet structure. The two doublets have the same chemical shift for ^{15}N (-35.6 ppm) and differ for the chemical shift of the ^1H (6.68 and 6.43 ppm, respectively). These signals pertain to the two aminic protons that are diastereotopic but become magnetically unequivalent only after coordination of the amino acid to the platinum atom. The ^{15}N chemical shift is also indicative of a trans chlorine atom.[34] The trifluoromethyl signal is more shielded in the coordinated amino acid ($\delta = -77.26$ ppm) than in the free ligand ($\delta = -74.81$ ppm; ^{19}F NMR spectra recorded in H_2O/D_2O).

The cationic complexes **2**−**5** containing, besides the N,O-chelated amino acid, neutral N-donor ligands were prepared from [$PtAm_2(NO_3)_2$] and the pure enantiomers of α-Tfm-Ala as shown in Scheme 3. The obtained nitrate salts were very soluble in water, highly hygroscopic, and difficult to obtain in crystalline form.

The [$Pt(NH_3)_2$(α-Tfm-Ala)]$^+$ complexes **2*S*** and **2*R*** showed a peak at *m/z* = 385 in the positive MALDI TOF spectrum (water solution), which is assigned to the parent ion M^+ ($C_4H_{11}N_4O_5F_3Pt$). As already observed for compound **1**, also for compound **2** the coordination to platinum causes a deshielding of the methyl protons (1.87 versus 1.72 ppm in the free ligand) and a shielding of the trifluoromethyl group (-75.88 versus -74.81 ppm in the free ligand) of the amino acid. The N,O-chelation

is supported by the ^{195}Pt NMR spectrum, showing a single broad signal ($\delta = -2180$ ppm) in the range typical for a N_3O set of donor atoms (the presence of three ^{14}N donor atoms is responsible for the remarkable broadening of the platinum peak). The 2D ^{1}H−^{15}N HSQC spectrum shows three signals. Two signals having very similar ^{15}N/^{1}H chemical shifts (^{15}N/^{1}H = −68.9/4.33 ppm and −68.5/4.26 ppm) are assigned to the amine group of (*S*)-α-Tfm-Ala and to the trans NH_3 ligand, respectively. The third signal (^{15}N/^{1}H = −88.4/4.29 ppm) is assigned to the NH_3 ligand trans to the carboxylic oxygen atom. The given assignment is in accord with literature data pertaining to the analogous glycine complex $[Pt(NH_3)_2(Gly)]^+$.[14] The presence of a doublet of doublets for the aminic protons of α-Tfm-Ala in compound **1** and of only a singlet in compound **2** was rather surprising because also for compound **2** we expected a diastereotopic splitting of the two aminic protons caused by the presence of different substituents (CF_3 and CH_3) on the adjacent α carbon atom. A possible explanation for the absence of such a splitting could be that the amino acid, while biscoordinated in **1**, is monocoordinated in **2**, and therefore, a fast rotation about the N−C bond renders the inequivalence of the aminic protons nonobservable in the NMR. This hypothesis, however, contrasts with the ^{1}H and ^{19}F chemical shifts of the amino acid, which are practically coincident for the two types of complexes. Furthermore, the Pt−O bond of a coordinated carboxylic group is expected to be stronger in **2** (trans to an N donor) than in **1** (trans to a chloride). Another possibility is that the acidity of the aminic protons, already high because of the presence of a CF_3 substituent on the adjacent carbon atom (for α-Tfm-Ala the estimated pK_a values are 1.2 for the carboxylic group and 5.4 for the protonated aminic group), is further increased by the N-coordination to a platinum center bearing a formal positive charge. Fast exchange of each NH_2 proton between the two diastereotopic positions would lead to signal averaging in the HSQC spectrum. In the case of compound **1**, the platinum center carrying a formal negative charge drains less charge from the coordinated nitrogen atoms with consequent lower acidity of the NH_2 protons. This interpretation of the NMR data is fully supported by the results obtained with the following compound **3**.

The complexes $[Pt(en)(S\text{-}α\text{-Tfm-Ala})]^+$ and $[Pt(en)(R\text{-}α\text{-Tfm-Ala})]^+$ (**3*S*** and **3*R***, respectively; en = ethylendiamine) showed a peak at *m/z* = 411 (with reference to the most abundant peak in the isotopic cluster) in the positive MALDI TOF spectrum (water solution) assigned to the parent ion M^+ ($C_6H_{11}F_3N_3O_2Pt$). Two signals in the C−H region of the ^{1}H NMR spectrum are assigned to the amino acid methyl (1.85 ppm) and to the four methylene protons of ethylendiamine (singlet at 2.60 ppm). The latter signal shows broad satellites due to coupling with ^{195}Pt. Similar to compounds **1** and **2**, for compound **3** the trifluoromethyl signal undergoes a shielding ($\Delta\delta = -1.07$ ppm, Table 1) after coordination. The NMR signal of the ^{195}Pt nucleus (−2470 ppm) is rather broad and falls in the typical range of an N_3O coordination environment (Figure 2).

The 2D ^{1}H−^{15}N HSQC spectrum (Figure 3) shows three sets of signals. The first set (^{15}N/^{1}H cross-peaks at −49.3/5.61 and −49.3/5.47 ppm) is an ill-resolved

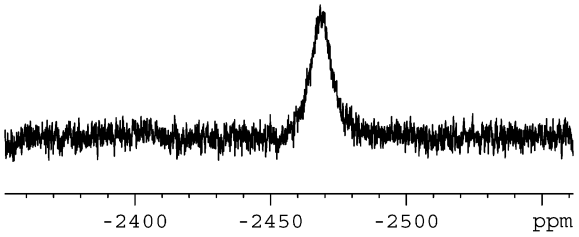


Figure 2. ^{195}Pt NMR in D_2O of $[Pt(en)(S\text{-}α\text{-Tfm-Ala})]^+$.

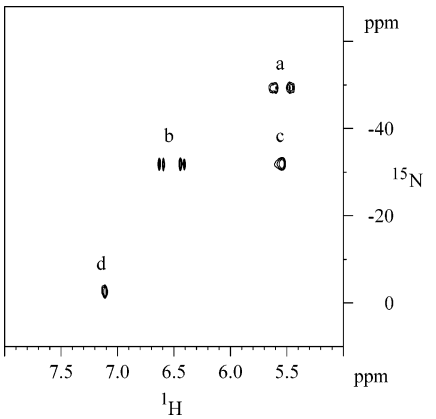


Figure 3. ^{1}H−^{15}N HSQC (H_2O/D_2O 90/10 v/v; pH 4.54) of $[Pt(en)(S\text{-}α\text{-Tfm-Ala})]^+$: (a) signal for $-NH_2$ of en trans to oxygen; (b) signal for $-NH_2$ of en trans to nitrogen; (c) $-NH_2$ signal for coordinated (*S*)-α-Tfm-Ala; (d) $-NH_2$ signal for free (*S*)-α-Tfm-Ala.

Table 1. Changes of $-CH_3$ and $-CF_3$ Chemical Shifts (with Respect to the Free Amino Acid) for $[PtX_2(α\text{-Tfm-Ala})]^y$ Complexes

X_2 (complex)	y	$\Delta\delta$ ^{19}F (ppm)	$\Delta\delta$ ^{1}H (ppm)
Cl_2 (**1**)	−1	−2.45	0.17
$(NH_3)_2$ (**2**)	+1	−1.07	0.15
en (**3**)	+1	−1.08	0.12
phen (**4**)	+1	−0.86	0.27
Me_2phen (**5**)	+1	−0.26	0.15

doublet of doublets assigned to the NH_2 group of ethylendiamine trans to the carboxylic group. The second set of cross-peaks (^{15}N/^{1}H at −31.7/6.60 and −31.7/6.42 ppm) is a better resolved doublet of doublets assigned to the NH_2 group of en trans to the aminic functionality of the amino acid. The remaining signal (^{15}N/^{1}H at −32.1/5.55 ppm) is assigned to the NH_2 group of the (*S*)-α-Tfm-Ala ligand. In the spectrum a ^{15}N/^{1}H cross-peak at −2.5/7.11 ppm is also visible, belonging to a small amount of the free amino acid added to the solution for comparison purpose. Three conclusions can be drawn from this 2D ^{1}H−^{15}N HSQC spectrum, which are in full agreement with the previous interpretation of the NMR data for compound **2**: (i) The presence of a doublet of doublets for each aminic group of ethylendiamine clearly indicates that the α-Tfm-Ala ligand is chelated. In the absence of chelation the inequivalence of the two protons would not be observable in the NMR spectrum. (ii) The presence of a singlet signal for the aminic group of (*S*)-α-Tfm-Ala cannot be explained with a smaller dispersion of chemical shifts for these NH_2 protons compared to those of en, since the former protons are closer to the center of dissymmetry (the α carbon bearing the CF_3 and CH_3 substituents) than the NH_2 groups of en; therefore, a greater acidity of the aminic protons of the coordinated amino acid allowing

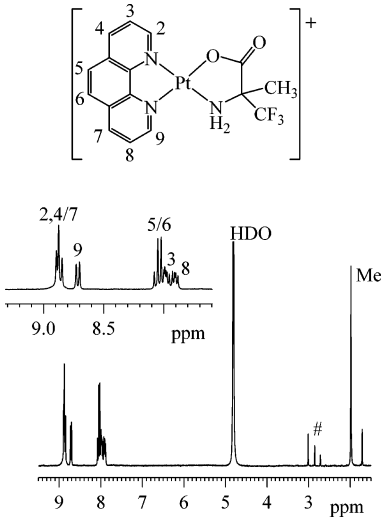


Figure 4. ^{1}H NMR spectrum (D_2O, pH* 6.22) of [Pt(phen)-(*S*-α-Tfm-Ala)]$^+$ (**4*S***) together with phenanthroline numbering scheme. Me indicates the (*S*)-α-Tfm-Ala methyl group. HDO indicates the residual solvent peak, and # indicates impurities. The phen proton signals are marked with the corresponding proton number in the expansion of the aromatic region.

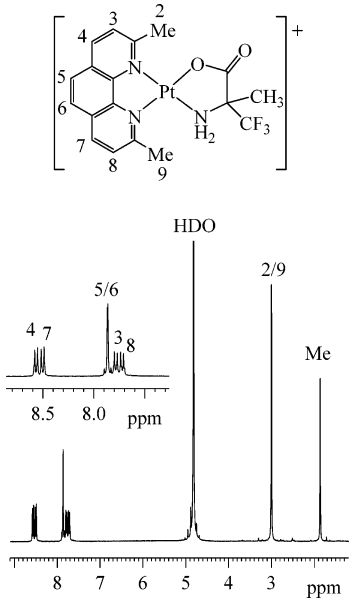


Figure 5. ^{1}H NMR spectrum (D_2O, pH* 4.50) of [Pt(Me_2phen)-(*S*-α-Tfm-Ala)]$^+$ (**5*S***) together with the phenanthroline numbering scheme. Me indicates the (*S*)-α-Tfm-Ala methyl group. HDO indicates the residual solvent peak, and Me_2phen proton signals are marked with the corresponding proton number in the expansion of the aromatic region.

their exchange between the two diastereotopic positions with consequent signal averaging in the HSQC spectrum is the only likely explanation. (iii) The electrophilicity of the metal center carrying a formal positive charge is sufficient to cause scrambling of protons for the NH_2 protons of the amino acid already sensitized by the presence of the trifluoromethyl substituent but not to cause scrambling of the NH_2 protons of en.

Also, the complex [Pt(phen)(*S*-α-Tfm-Ala)]$^+$ (**4*S***; phen = 1,10-phenanthroline) was characterized by NMR. As expected, the singlet signal of the methyl protons is deshielded (1.98 ppm) while that of the trifluoromethyl group is shielded (−75.73 ppm) if compared to those of the free amino acid. Two multiplets at 7.97 and 7.90 ppm are assigned to the protons 3 and 8 of the phenanthroline. Protons 5 and 6 give rise to an AB system with chemical shifts at 8.08 and 7.99 ppm and J_{AB} of 8.78 Hz. Protons 2 and 9 give two doublets centered at 8.88 and 8.71 ppm, the former doublet overlapping with a doublet centered at 8.86 ppm and belonging to protons 4/7 (Figure 4). The most deshielded proton of the phenanthroline ring is the one in position 2, cis to the carboxylic oxygen atom (the characterization was accomplished by a 2D ROESY experiment; data not shown).

The nitrate salt of the complex [Pt(Me_2phen)(*S*-α-Tfm-Ala)]$^+$ (**5*S***) turned out to be less hygroscopic than the previous ones and was characterized also by IR spectroscopy. The IR spectrum revealed the presence of a strong band at 1384 cm^{-1} assigned to the nitrate ion, a strong band at 1698 cm^{-1} assigned to the C=O group (1683 cm^{-1} in free *S*-α-Tfm-ala), and a strong band at 1204 cm^{-1} assigned to the stretching of the CF_3 group (1167 cm^{-1} in the free amino acid). The ^{1}H NMR spectrum is shown in Figure 5. The methyl signal of the coordinated amino acid falls at 1.86 ppm, while the signals of the methyl substituents in positions 2 and 9 of the phenanthroline fall at 2.99 and 3.00 ppm and are very close to each other. The aromatic protons, in the order of decreasing shielding, are 3 and 8 (two doublets at 7.71 and 7.75 ppm, respectively), 5 and 6 (AB system with chemical shift very close to 7.86 ppm), and 4 and 7 (doublets at 8.49 and 8.56 ppm, respectively).

As usual for this family of compounds, the signal of the trifluoromethyl substituent (−76.68 ppm) is slightly shielded with respect to the free amino acid (−76.42 ppm).

The changes in the ^{19}F chemical shifts of the trifluoromethyl group (Table 1) appear to be modulated by the overall charge of the complex. Greater shielding is associated with the anionic species, while the cationic species exhibit lower shielding. The shielding is the lowest in the Me_2phen complex probably because of an additional effect due to the steric distortions imposed by the bulky methyl substituents at C(2) and C(9). It has been shown that Me_2phen, to relieve the steric interactions between the methyl substituents of the phenanthroline and the cis ligands on the platinum atom, undergoes a bow-like distortion (dihedral angles between the average planes of the pyridine rings in the range 14−17°) and a rotation (about the axis connecting the two coordinated nitrogen atoms) of the phenanthroline plane with respect to the coordination plane (dihedral angle between the two planes in the range 38−44°).[35−37] In the case of the unsubstituted phenanthroline, the ligand is planar and lays in the coordination plane.[38]

The variations in ^{1}H chemical shifts for the methyl group of *S*-α-Tfm-Ala are generally smaller than those observed for the ^{19}F chemical shifts and more homogeneous (Table 1). The change in chemical shift is slightly greater for the phen complex in which the aromatic ring system is coplanar with the coordination plane.

We can conclude that the chelating ability of *S*-α-Tfm-Ala toward platinum is similar to that of natural amino acids; however, the greater acidity of *S*-α-Tfm-Ala with respect to the natural counterpart [alanine, pK_a(COOH)

Table 2. Cytotoxicity (μM) of the Platinum Complexes with α-Tfm-Ala toward Different Human Tumor Cell Lines, in Comparison with Cisplatin

	cytotoxicity (μM)				
compd	NCI-H460	HCT-116	MCF-7	HUVEC	A549
1*R*	56.2 ± 5.9	ne[a]	ne[a]	ne[a]	68.7 ± 4
1*S*	85.1 ± 5.6	ne[a]	ne[a]	ne[a]	100
2*R*	2.9 ± 0.4	30	48.6 ± 10	23 ± 6.4	28.1 ± 1.4
2*S*	19.3 ± 1.6	>50	73.1 ± 9	42 ± 0.3	11.1 ± 2.0
3*R*	147 ± 22	ne[a]	ne[a]	ne[a]	100
3*S*	165 ± 46	ne[a]	ne[a]	ne[a]	>100 (31%)
cisplatin	0.6 ± 0.08	2.5 ± 0.3	>3.3	5.4 ± 1.1	2 ± 0.2

[a] ne = not evaluated.

= 2.3 and p$K_a(NH_3^+)$ = 9.9][39] is not without effect. Depending on the electrophilicity of the metal core, the two diastereotopic aminic protons of the amino acid undergo scrambling (cationic complexes) or remain stable (anionic compounds). The aminic protons of en remain stable (do not exchange between the two diastereotopic positions) even in the cationic complexes. This study also revealed how the electrophilicity of the metal core strongly depends on the formal charge of the complex.

Pharmacological Assays. Biological tests were carried out only on compounds **1**−**3** because compounds **4** and **5** containing phenanthrolines are characterized by a peculiar reactivity toward glutathione (leading sometimes to displacement of the dinitrogen ligand), which needs to be investigated before proceeding to further tests. The cytotoxicity of the novel compounds **1**−**3** was first evaluated against two tumor lines responsive to the reference compound, cisplatin (NCI-H460 and A549, both human non-small-cell lung carcinomas). The most interesting result was obtained from complex **2*R***, which showed a potency only 5-fold lower than that of cisplatin (IC_{50} = 2.9 and 0.6 μM, respectively; 24 h exposure time; Table 2) toward NCI-H460 cells. Rather interestingly the **2*S*** enantiomer was ∼6 times less active. The same pair of compounds (**2*R*** and **2*S***) was also active against the A549 tumor cell line; however, the activity was slightly smaller and, furthermore, an inversion of activity was observed with the **2*S*** enantiomer being now more active than the **2*R*** counterpart (11.1 and 28.1 μM, respectively). The other compounds were less active toward the above-mentioned cell lines and were excluded from the following experiments on additional human tumor cell lines: HCT-116 colon carcinoma, MCF-7 breast carcinoma, and endothelial cells HUVEC. Quite disappointingly the lead compounds **2*R*** and **2*S*** did not show activity comparable to that of cisplatin (IC_{50} ranged from 23 to 48.6 μM for **2*R***).

DNA flow cytometric analysis was performed in order to have insight into the mechanism of action of the lead compounds. These complexes are cationic, while cisplatin is neutral. The results indicated that the cycle of NCI-H460 tumor cells, exposed to **2*R*** or **2*S*** at a concentration equal to twice the IC_{50}, was not perturbed compared to that of tumor cells treated with the vehicle alone. In contrast, tumor cells exposed to cisplatin for 24 h were blocked in the G_2/M phase (Table 3). At this stage of the investigation we can only conclude that the mechanism of action of **2*R*** and **2*S*** appears to be different from that of cisplatin, and this is also confirmed by the inability of the novel compounds to induce apoptosis (Table 3).

Table 3. FACS Analysis of NCI-H460 Tumor Cells Exposed for 24 h to **2*R***, **2*S***, and in Another Experiment to Cisplatin[a]

	$G_{0/1}$ (%)	S (%)	G_2/M (%)	Apo (%)
untreated	52.9	36.7	10.4	0.9
2*R*	50.1	36.2	13.7	0.7
2*S*	49.9	35.8	14.4	0.8
untreated	62.7	30.9	6.4	1.0
cisplatin	33.3	29.0	37.7	2.1

[a] In all cases the doses were equal to 2 × IC_{50}.

Although the overall results of the in vitro experiments are not exciting (with the exception of complex **2*R***, which appears to be worth further biological investigation), the observation that the activity of the complexes depends rather strongly upon the configuration of the α-Tfm-Ala ligand is rather interesting. With reference to compound **2**, the *R* enantiomer is more active than the *S* counterpart in most cases except with the A549 cell line where the *S* enantiomer is more active. This is quite an intriguing result, and further experiments are needed before an exhaustive correlation between pharmacological activity and chirality of the bound amino acid can be established. At the beginning of this investigation we expected that the *S* enantiomer (i.e., the *L* one) could be more active than the *R* enantiomer, since it could take advantage of the transport systems across the cell membrane of natural amino acids.[40] The results do not appear to support this.

Conclusions

In this work, a new series of platinum complexes having, as common ligand, a pure enantiomer of the fluorinated amino acid α-Tfm-Ala have been synthesized and characterized. The complexes were very soluble in water and highly hygroscopic, with the only exception being complex **5*S***. The fluorinated amino acid showed good coordinating ability toward platinum, and the acidity of the protons on the coordinated nitrogen appears to be strongly influenced by the formal charge of the complex.

Complexes **1**−**3** have been tested for their in vitro cytotoxicity against different human tumor cell lines. Rather interestingly, complexes **2*R*** and **2*S*** showed activities comparable to that of cisplatin (especially **2*R***). Moreover, the activity appears to depend on the chirality of the amino acid, indicating that a precocious release of the fluorinated amino acid is rather unlikely. Furthermore, the results of the flow cytometric experiments (no arrest in G_2/M phase, which instead is observed in the case of cisplatin) and the absence of apoptosis in the treated tumor cells demonstrate that the mechanism of action of the novel compounds is quite different from that of cisplatin.

In conclusion, substitution of the α-Tfm-Ala amino acid for the two chlorine atoms in cisplatin greatly increases the water solubility of the complex while preserving the cytotoxic activity, which appears to imply a mechanism different from that of cisplatin. This result is worth further investigation in view of the development of a new generation of platinum drugs with improved pharmacokinetic properties and able to overcome the intrinsic or induced resistance of several tumors toward cisplatin.

Material and Methods

Starting Materials. The amino acids *R*- and *S*-α-Tfm-Ala (2*R*- and 2*S*-2-amino-3,3,3-trifluoro-2-methylpropanoic acid) were synthesized as previously described.[24,32] The reagents $K_2[PtCl_4]$, $[Pt(NO_3)_2(NH_3)_2]$,[41] $[Pt(NO_3)_2(en)]$[42] (en = ethylendiamine), $[PtCl_2(phen)]$[43] (phen = 1,10-phenanthroline), and $[PtCl_2(Me_2phen)]$[36] (Me_2phen = 2,9-dimethyl-1,10-phenanthroline) were synthesized according to previously published procedures.

Instrumental Measurements. pH measurements were conducted using a Crison Micro-pH model 2002 apparatus equipped with a 3 mm microelectrode and calibrated using Crison standard buffers at pH 4.00, 7.02, and 9.00. The reported pH values, when measured in D_2O, are indicated as pH* and correspond to the direct readings from the instrument, without correction for the effect of deuterium on the glass electrode.[44]

IR spectra were recorded on a Perkin-Elmer Spectrum One spectrophotometer using KBr as solid support for pellets. Elemental analyses were performed using a Carlo Erba elemental analyzer model 1106 instrument.

The ^1H, ^{195}Pt, and $^1H-^{15}N$ HSQC NMR spectra were acquired using a Bruker Avance DRX 300 MHz instrument. Standard pulse sequences were used for ^1H and $^{195}Pt\{^1H\}$ 1D spectra. The COSY, TOCSY, and $^1H-^{15}N$ HSQC experiments were performed using gradient selected versions of the pulse programs. ^1H chemical shifts were referenced to internal TSP. ^{195}Pt chemical shifts were referenced to external $K_2[PtCl_4]$ in D_2O fixed at −1615 ppm. ^{15}N chemical shifts were referenced to external 1 M $^{15}NH_4Cl$ in 1 M HCl.

^{19}F NMR spectra were acquired with a Varian VX Mercury 300 MHz spectrometer, using $CFCl_3$ as the internal standard.

MALDI-TOF mass spectrometric analyses were performed on an Applied Biosystems Voyager DE-PRO MALDI-TOF mass spectrometer equipped with an N_2 laser (λ = 337 nm, impulse = 3 ns), a flight tube of 1.3 m length in linear mode and 2.0 m in reflector mode, and a digitizer Voyager-DE 500 MHz Signatec. Instrumental conditions were the following: matrix, α-cyano-4-hydroxycinnamic acid in acetonitrile/water 50/50; sample preparation, 1 μL of a 20 μM water solution of the sample, mixed with 10 μL of a 50/50 acetonitrile/water solution of α-cyano-4-hydroxycinnamic acid (10 mg/mL); mode of operation, reflector; accelerating voltage, 25 000 V; grid voltage, 80%; delay time, 100 ns; guide wire, 0.001%.

Preparation of the Platinum Complexes. $[Pt(NO_3)_2(Me_2phen)]$. A solution containing $[PtCl_2(Me_2phen)]$ (200 mg, 0.420 mmol) dissolved in dimethylformamide (70 mL) was treated with $AgNO_3$ (143 mg, 0.840 mmol) dissolved in a minimum amount of water. After being stirred in the dark for 12 h at room temperature, the mother solution was filtered through Celite to remove AgCl. The filtered yellow solution was treated with diethyl ether (450 mL), which caused the formation of a creamy yellow precipitate. The precipitate was collected by filtration of the solution, washed with ether, and dried under vacuum. Yield = 70% (160 mg). Anal. ($C_{14}H_{12}N_4O_6Pt$) C, H, N.

$[Pt(NO_3)_2(phen)]$. The procedure is identical to that used for $[Pt(NO_3)_2(Me_2phen)]$ starting from $[PtCl_2phen]$. Anal. ($C_{12}H_8N_4O_6Pt$) C, H, N.

K[$PtCl_2$(*S*-α-Tfm-Ala)] (1*S*) and K[$PtCl_2$(*R*-α-Tfm-Ala)] (1*R*). The preparation procedure was identical for both the *S* and *R* enantiomers of α-Tfm-Ala. In a typical experiment $K_2[PtCl_4]$ (50 mg, 0.121 mmol) was dissolved in 2 mL of H_2O. α-Trifluoromethylalanine (19 mg, 0.121 mmol) was added to the solution, which was kept under stirring at 50 °C. NMR spectra were recorded from time to time by evaporating to dryness small aliquots of the mother solution and redissolving the solid residue in D_2O. The pH was maintained at a constant value of ∼6.0 by additions of KOH (0.02 M solution) when required. After completion of the reaction (approximately 1 week) the solution was freeze-dried and the residue was treated with absolute ethanol to separate the product from insoluble KCl. After filtration, the solvent was evaporated to dryness and the product of the reaction, a yellow hygroscopic solid, was kept in a Shlenk tube under argon atmosphere. Yield = 54% (30 mg) for **1*R*** and 70% (39 mg) for **1*S***. Anal. ($C_4H_5NCl_2F_3KNO_2Pt$) C, H, N. MS MALDI-TOF: M^- (% relative abundance) = 420 (22), 421 (22), 422 (28), 423 (15), 424 (13). NMR data: ^1H, δ = 1.89 ppm (3H, s); ^{19}F, δ = −77.26 ppm; ^{195}Pt, δ = −1627 ppm (br) (D_2O; pH* 6.00). $^{15}N-^1H$ HSQC, δ $^{15}N/^1H$ = −35.6/6.68 and −6.68/6.43 ppm (H_2O/D_2O, 90:10 v/v, pH 4.05).

$[Pt(NH_3)_2$(*S*-α-Tfm-Ala)$](NO_3)$ (2*S*) and $[Pt(NH_3)_2$(*R*-α-Tfm-Ala)$](NO_3)$ (2*R*). The preparation procedure was identical for both enantiomers of α-Tfm-Ala. In a typical experiment $[Pt(NO_3)_2(NH_3)_2]$ (50 mg, 0.141 mmol) was dissolved in 3 mL of H_2O and the solution was brought to 50 °C. After addition of α-trifluoromethylalanine (22 mg, 0.141 mmol), the yellow solution was kept under stirring (always at 50 °C), readjusting the pH to the initial value of 6.0 by addition of KOH (0.02 M solution) when needed. The completeness of the reaction was revealed by no further change in the pH value (approximately 1 week). The final pale-yellow solution was freeze-dried. The dark-green hygroscopic solid, containing some KNO_3, was treated with pure ethanol (20 mL), the suspension was filtered, and the filtrate was taken to dryness by evaporation of the solvent under vacuum. The product of the reaction, a yellow hygroscopic solid, was collected and kept in a Shlenk tube under an argon atmosphere. The yield was 65% (41 mg) for **2*R*** and 65% (40 mg) for **2*S***. Anal. ($C_4H_{11}N_4F_3O_5Pt$) C, H, N. MS MALDI-TOF: M^+ (% relative abundance) = 384 (35), 385 (27), 386 (30), 387 (2), 388(6). NMR data: ^1H, δ = 1.87 ppm (3H, s); ^{19}F, δ = −75.88 ppm; ^{195}Pt, δ = −2180 ppm (br) (D_2O; pH* 4.50). $^{15}N-^1H$ HSQC, δ $^{15}N/^1H$ = −88.4/4.30, −68.9/4.33, and −68.5/4.26 ppm (H_2O/D_2O, 90:10 v/v, pH 4.54).

$[Pt(en)$(*S*-α-Tfm-Ala)$](NO_3)$ (3*S*) and $[Pt(en)$(*R*-α-Tfm-Ala)$](NO_3)$ (3*R*). The preparation procedure was identical for both enantiomers of α-Tfm-Ala and similar to that of compound **2**. Briefly $[Pt(NO_3)_2(en)]$ (50 mg, 0.132 mmol) was dissolved in 3 mL of H_2O, and the solution brought to 50 °C. After addition of α-trifluoromethylalanine (6.0 mg, 0.038 mmol), the solution was kept under stirring at 50 °C and the pH was maintained at a constant value of ∼6.0 by adding, when needed, KOH (0.02 M). The progress of the reaction was followed from time to time by NMR spectroscopy (small aliquots of the mother solution were evaporated to dryness, and the solid residue was redissolved in D_2O and monitored by ^1H NMR). After completion of the reaction (∼1 week), the products were isolated as hygroscopic yellow solids, as already reported for complexes **1** and **2**. The yield was 80% (48 mg) for **3*R*** and 70% (45 mg) for **3*S***. Anal. ($C_6H_{13}N_4F_3O_5Pt$) C, H, N. MS MALDI-TOF: M^+ (% relative abundance) = 410 (23), 411 (33), 412 (29), 413 (9), 414 (6). NMR data: ^1H, δ = 2.60 (4H, pseudo triplet with platinum satellites, $^3J_{Pt-H}$ = 45.2 Hz) and 1.85 (3H, s) ppm; ^{19}F, δ = −75.89 ppm; ^{195}Pt, δ = −2470 ppm (br) (D_2O, pH* 6.00). $^{15}N-^1H$ HSQC, δ $^{15}N/^1H$ = −49.3/5.61 and −49.3/5.47, −31.7/6.60 and −31.7/6.42, −32.1/5.55, and −2.5/7.11 ppm (H_2O/D_2O, 90:10 v/v; pH 4.18).

$[Pt(phen)$(*S*-α-Tfm-Ala)$](NO_3)$ (4*S*). In an NMR tube, $[Pt(NO_3)_2(phen)]$ (5 mg, 0.010 mmol), suspended in D_2O (1 mL), was treated with *S*-α-trifluoromethylalanine (1.57 mg, 0.010 mmol), and the temperature was maintained at 50 °C while keeping the pH* to the initial value of ∼6.0 by addition of 0.02 M KOD. The product of the reaction, not isolated, was characterized by NMR spectroscopy. NMR data: ^1H, δ = 8.88 (2H, d, $^3J_{H-H}$ = 5.3 Hz), 8.86 (1H, d, $^3J_{H-H}$ = 8.34 H), 8.71 (1H, d, $^3J_{H-H}$ = 8.34 Hz), 8.08−7.99 (2H, dd, $^3J_{H-H}$ = 8.78 Hz), 7.97 (1H, m), 7.90 (1H, m), and 1.98 (3H, s) ppm; ^{19}F, δ = −75.73 ppm (D_2O, pH* 6.22).

$[Pt(Me_2phen)$(*S*-α-Tfm-Ala)$](NO_3)$ (5*S*). A suspension of $[Pt(NO_3)_2(Me_2phen)]$ (22.5 mg, 0.053 mmol; Me_2phen = 2,9-dimethyl-1,10-phenanthroline) in D_2O (1 mL) was treated with *S*-α-trifluoromethylalanine (8.4 mg, 0.053 mmol). The suspension was transferred into an NMR tube, and the temperature was maintained at 50 °C while adjusting the pH* to the initial value of 6.0 with KOD (0.02 M solution). The reaction was monitored by NMR spectroscopy, and after complete disappearance of the signals of the starting species (approximately

one week), the yellow precipitate was isolated from the NMR tube and characterized. Anal. ($C_{18}H_{15}N_4F_3O_4Pt$) C, H, N. NMR data: ^1H, δ = 8.56 (1H, d, $^3J_{H-H}$ = 8.39 Hz), 8.49 (1H, d, $^3J_{H-H}$ = 8.39 Hz), 7.86 (2H, AB system with very close chemical shifts, $^3J_{H-H}$ = 8.47 Hz), 7.75 (1H, d, $^3J_{H-H}$ = 8.39 Hz), 7.71 (1H, d, $^3J_{H-H}$ = 8.39 Hz), 3.00 (3H, s), 2.99 (3H, s), and 1.86 (3H, s) ppm; ^{19}F, δ = −76.68 ppm (D_2O, pH* 4.50).

Pharmacological Assays. Tumor Cell Lines. NCI-H460 (human non-small-cell lung cancer) and MCF-7 (human-hormone-dependent breast cancer cell lines) were grown in RPMI 1640 containing 10% fetal bovine serum and 50 μg/mL gentamycin sulfate. HCT-116 cells (human colon cancer cells) were grown in McCoy's containing 10% fetal bovine serum and 50 μg/mL gentamycin sulfate. A549 (NSCLC) cells were grown in Ham's F12 containing 10% fetal bovine serum and 50 μg/mL gentamycin sulfate. HUVEC cells (human endothelial cells) were grown in EBM2 (Clonetics) containing EGM-2 SingleQuots (Clonetics).

To test the effect of the compounds on cell growth, cells were seeded in 96-well tissue culture dishes (Corning) and were allowed to attach and recover for at least 24 h. Different concentrations of each compound were then added to the wells, obtaining the following final concentrations in the wells: 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56 μM. The plates were incubated for 24 h. The compounds were removed, and after 48 h of recovery, the number of surviving cells was determined by staining with the sulforhodamine B test.[45]

For the flow cytometric study, 1×10^6 cells were seeded in 100 mm dishes and the next day were treated with the platinum drugs for 24 h at the IC_{50} and $2 \times IC_{50}$ doses. Successively, cells were harvested with trypsin/EDTA and pooled with the respective supernatants. After two washings in PBS, cells were fixed in cold 70% ethanol and stored at 4 °C. On the day of analysis, ethanol was removed by centrifugation and cells were washed twice with PBS and treated with RNAse (75 kU/mL) for 30 min at 37 °C. Propidium iodide (PI) was finally added (50 μg/mL) to stain the cellular DNA, and samples were processed by a FACScan. The 2×10^4 cells were acquired for each sample using the CELLQuest software and recording propidium iodide (PI) FL-2 fluorescence.

For FACS analysis, apoptosis was evaluated by measuring the percentage of cells with hypodiploid DNA content (sub-$G_{1/0}$ population) with the CELLQuest program, while cell cycle analysis was performed with the ModFit software.

Sample Preparation. All complexes were dissolved in water. Cisplatin (Platinex) was the formula preparation and was diluted in the medium culture.

Data Analysis. To analyze the cytotoxicity of the compounds, IC_{50} values were evaluated by using the ALLFIT program.

Acknowledgment. The authors thank Università degli Studi di Bari, Università dell'Aquila (ex 60% funds), the Italian "Ministero dell'Istruzione, Università e Ricerca (MIUR)" (PRIN 2004, Grant No. 2004059078_006), and the EC (COST Chemistry Projects D20/0001/2000 and D20/0003/01) for support. The authors are also grateful to Dr. Francesca Attanasio, Università dell'Aquila, for the execution of the MALDI-TOF analyses.

Supporting Information Available: Table S1 listing the results from elemental analysis of [Pt(NO_3)$_2$(Me$_2$phen)], [Pt(NO_3)$_2$(phen)], K[PtCl$_2$(α-Tfm-Ala)] (**1*S*** and **1*R***), [Pt(NH_3)$_2$(α-Tfm-Ala)](NO_3)·2H_2O (**2*S*** and **2*R***), [Pt(en)(α-Tfm-Ala)](NO_3)·2H_2O (**3*S*** and **3*R***), and [Pt(Me$_2$phen)(*S*-α-Tfm-Ala)](NO_3)·H_2O (**5*S***). This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0504003