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A Study on the Inhibition of DiHydroFolateReductase (DHFR) from *Escherichia coli* by Gold(I) Phosphane Compounds. X-ray Crystal Structures of (4,5-dichloro-1H-imidazolate-1-yl)-triphenylphosphane-gold(I) and (4,5-dicyano-1H-imidazolate-1-yl)-triphenylphosphane-gold(I).

Rossana Galassi,[,#] Camille Simon Oumarou,[#] Alfredo Burini,[#] Alessandro Dolmella,[°] Daniela Micozzi,[§] Silvia Vincenzetti, [§] Stefania Pucciarelli.[§]*

[#] School of Science and Technology, Chemistry Division, University of Camerino, Via S. Agostino, 1, 62032 Camerino, Italy

°Department of Pharmaceutical and Pharmacological Sciences, Università di Padova, Via Marzolo 5, 35131 Padova, Italy

§ School of Biosciences and Biotechnology, University of Camerino, Via Gentile III da Varano, 1, 62032 Camerino, Italy

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Abstract.

An unprecedented study on the inhibitory activity of a class of phosphane gold(I) complexes on *E. coli* DiHydroFolate Reductase (DHFR) is reported. The gold(I) complexes considered in this work consist of azolate or chloride ligands and phosphane as co-ligands. The ligands have been functionalized with polar groups (-COOH, $-COO^-$, NO_2, Cl, CN), to obtain better solubility in polar media. Neutral, anionic and cationic gold(I) complexes have been tested as DHFR inhibitors by means of a continuous direct spectrophotometric method. X-ray structural characterizations were performed on ((triphenylphosphine)-gold(I)-(4,5-dicyanoimidazolyl-1H-1yl) and the second on the analog (triphenylphosphine)-gold(I)-(4,5-dichloroimidazolyl-1H-1yl). The inhibition constants obtained from the enzymatic tests range from 20 μM to 63 nM (auranofin) and are such to promote these compounds as potential DHFR inhibitors.

Introduction.

Dihydrofolate reductase (DHFR), an ubiquitous enzyme in all eukaryotic and prokaryotic cells, is of pivotal importance in biochemistry and medicinal chemistry. DHFR catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF), using NADPH as a coenzyme. THF is required for the

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de novo synthesis of purines, aminoacids and thymidylate (TMP). Therefore, inhibition of DHFR activity, in the absence of salvage mechanisms, hampers the supply of TMP for DNA biosynthesis, and eventually leads to cell death. The ability of preferentially killing cells that replicate at a high rate is the basis of anti-cancer chemotherapy. Thus DHFR has long been recognized as a drug target to treat cancer, and many DHFR inhibitors have been designed as antineoplastic drugs, such as methotrexate (MTX), representing the first class of antimetabolites to be introduced to the oncology clinic for the chemotherapeutic treatment of childhood Acute Lymphoblastic Leukemia (ALL), nearly 60 years ago.[1] Although most of the patients respond effectively to anti-folate treatment many of them relapse due to the development of resistance. Even if DHFR inhibitor methotrexate (MTX) remains a mainstay in single and combination cancer chemotherapy, there is a continuous search for new chemical entities with the potential to target this enzyme .[2] In fact blocking DHFR enzymatic activity is a key element in the treatment of many diseases in addition to cancer, like bacterial and protozoal infections and also opportunistic infections associated with AIDS (Pneumocystis Carinii Pneumonia, PCP), but also in various autoimmune diseases, e. g., rheumatoid arthritis.[3] The ability to inhibit enzymes is a well-known characteristic of gold(I) drugs; a number of putative mechanisms of action based on the interaction of gold(I) compounds with enzymes have been proposed during the “golden years” of anti-arthritis treatments research.[4] Recently, this study addressed other enzymes including cytochrome C and lysozyme ,[5] cysteine protease,[6] thioredoxin 1 and 2[7] and zinc finger protein PARP 1.[8] Although gold compounds have been well studied as anticancer drugs and many of them have been found very powerful,[9] cancer therapy based on gold drugs, from what we know, has never been seriously considered, mainly because the mechanism of action is not completely clear. However, as comparative cytotoxicity studies have shown,[10a] the presence of the metal center has its own meaning : the presence of metal activates biological effects on the organic ligand, which otherwise would be non-existent. Among all the possible enzymes, seleno-enzymes such as thioredoxine reductase (TrxR) have been extensively considered as the most likely molecular target. [7, 11] Some azolate gold(I) phosphane complexes synthesized by some of us have been tested on TrxR and on the structural analog disulfide glutathione reductase (GR), showing the strongest inhibition on the former. [10] These results promoted further enzymatic studies on this class of gold(I) compounds both in the structure/activity relationship and on the mechanism of action. For this latter aspect, and to evaluate the potential antimicrobial activity, a new crucial enzyme named Di-HydroFolate-Reductase (DHFR) was taken into consideration for the first time. The study was first led on a class

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of compounds whose anticancer activity on many cancer cell lines was previously ascertained, followed by a class of azolate gold(I) phosphane compounds related to the former but with enhanced polarity and hydrophilicity.

Results

Syntheses

In table 1 the investigated compounds are listed and named. Azolate gold(I) phosphane compounds **1**, **2**, **11** and **12** corresponding to the general formula of AzAuL (**1**, Az= 3,5-bis-trifluoromethyl-1H-pyrazolate-1-yl and L = PPh_3, triphenylphosphane; **2**, Az = (3,5-dinitro-1H-pyrazolate-1-yl) and L = PPh_3; **11**, Az = (4,5-dichloro-1H-imidazolate-1-yl) and L = PPh_3; **12**, Az = (1-benzyl-4,5-dichloro-2H-imidazolate-2-yl) and L = PPh_3 were synthesized as previously described.[10] In order to obtain better solubility and to study the inhibition activity as a function of the structure, compounds **3** - **10** and **13** - **17** were obtained by introducing polar functional groups both in the phosphane or/and in the azole ligands. Carboxylic or carboxylate functional groups in the phosphane ligand as well as chloro or cyano groups in the azolate ligand were considered. The role of the carboxylic group is to yield the PPh_3 ligand more polar and suitable for additional secondary interactions. Moreover, its presence creates a binding site allowing further functionalization and linkage to substrates, materials and / or GnPs.[12] We opted to use ortho- or para- substituted triphenylphosphane. Furthermore, measurement of logP in compound **11** showed the very negative value -0.548 (Shaked Flask Method, see experimental section) if compared to **1** and **2** (0.343 and 0.272, respectively). Then a similar imidazole was considered : the 4,5-dicyanoimidazole leading to compounds **15**, **16** and **17**.

Table 1. List of the compounds tested as DHFR inhibitors.

COMPOUND	NAME	
3,5-$Pz(CF_3)_2AuPPh_3$	[(3,5-bis(trifluoromethyl)pyrazolyl-1-yl)-(triphenylphosphane)gold(I)]	**1**
3,5-$Pz(NO_2)_2AuPPh_3$	[(3,5-bis(dinitro)pyrazolyl-1-yl)-(triphenylphosphane)gold(I)]	**2**
(4-COOH Ph)Ph_2PAuCl	[(4-benzoic acid-diphenylphosphane)-gold(I)chloride]	**3**

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(2-COOH Ph)Ph_2PAuCl	[(2-benzoic acid-diphenylphosphane)-gold(I)chloride]	**4**
[(4-COOH Ph)$Ph_2P]_2AuCl$	[Bis(4-benzoic acid-diphenylphosphane)-gold(I)chloride]	**5**
[(2-COOH Ph)$Ph_2P]_2AuCl$	[Bis(2-benzoic acid-diphenylphosphane)-gold(I)chloride]	**6**
(4-$COOEt_3NHPh)Ph_2PAuCl$	[triethylammonium][(4-benzoate-diphenylphosphane)-gold(I)chloride)]	**7**
(2-$COOEt_3NHPh)Ph_2PAuCl$	[triethylammonium][(2-benzoate-diphenylphosphane)-gold(I)chloride)]	**8**
[(4-$COOEt_3$NH Ph)$Ph_2P]_2AuCl$	Bis[triethylammonium][bis(4-benzoate-diphenylphosphane)-gold(I)chloride]	**9**
[(2-$COOEt_3NHPh)Ph_2P]_2AuCl$	Bis[triethylammonium][bis(2-benzoate-diphenylphosphane)-gold(I)chloride]	**10**
4,5-$Im(Cl)_2AuPPh_3$	[(4,5-dichloro-imidazolyl-1H)-(triphenylphosphane)-gold(I)]	**11**
1-Bz-4,5-$Im(Cl)_2$-C-$AuPPh_3$	[(1-benzyl-4,5-dichloro-imidazolyl-2yl)-(triphenylphosphane)-gold(I)]	**12**
4,5-$Im(Cl)_2$-N-$AuPPh_2$(4-COOHPh)	[(4,5-dichloro-imidazolyl-1yl)-(4-benzoic acid-diphenylphosphane)-gold(I)]	**13**
4,5-$Im(Cl)_2$-N-$AuPPh_2$(2-COOHPh)	[(4,5-dichloro-imidazolyl-1yl)-(2-benzoic acid-diphenylphosphane)-gold(I)]	**14**
4,5-Im(CN)2-N- $AuPPh_3$	[(4,5-dicyano-imidazolyl-1yl)-(triphenylphosphane)-gold(I)]	**15**
4,5-$Im(CN)_2$-N-$AuPPh_2$(4-COOHPh)	[(4,5-dicyano-imidazolyl-1yl)-(4-benzoic acid-diphenylphosphane)-gold(I)]	**16**
4,5-$Im(CN)_2$-N-$AuPPh_2$(2-COOHPh)	[(4,5-dicyano-imidazolyl-1yl)-(2-benzoic acid-diphenylphosphanegold(I)]	**17**

The syntheses of compounds **3** - **10** and **13** - **17** were carried out at room temperature and in the presence of air. The auration reaction was carried out with Me_2SAuCl in methanol with the subsequent work-up for the removal of Me_2S and the purification of the compounds by crystallization. The yields were medium / low due to weight losses during the hard purification work-up. By reacting compounds **3** and **4** with K_2CO_3 the corresponding potassium carboxylate salts were obtained, which resulted to be unstable both in solution and in the solid state and hence unsuitable for biological tests. On the other hand, stable tri-ethyl-ammonium carboxylate compounds (**7**, **8** and **9**, **10**) were isolated. Compounds **13 - 17** were synthesized as described in the experimental section by reaction of triphenylphosphane or 2- / 4-

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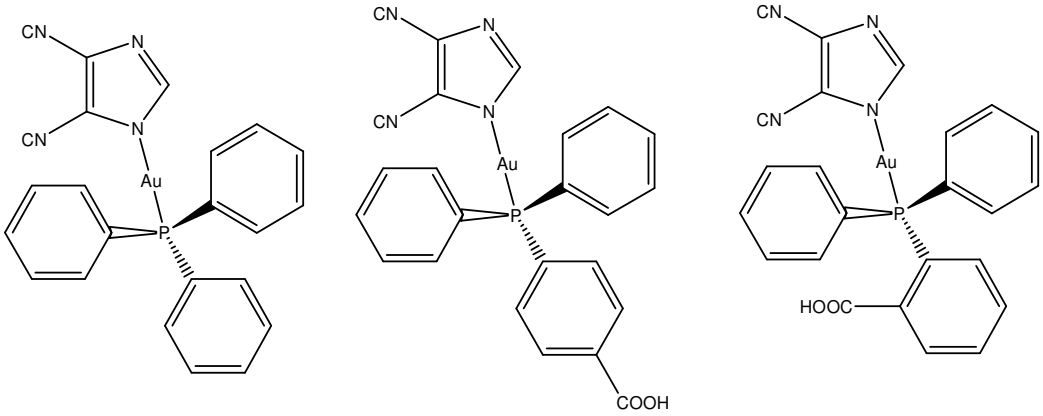
diphenylphosphinegold(I)chloride benzoic acids with the corresponding sodium 4,5-dichloro-imidazolate or 4,5-dicyano imidazolate salts, respectively (**15**, **16** and **17**).



Scheme. Schematic view of compounds **15**, **16** and **17.**

The IR spectroscopic characterization for compounds **3** - **10** and **13** - **17** showed the carbonyl stretching band ranging from 1717 to 1580 cm^{-1}, with red shifts for carboxylate with respect to carboxylic groups. The ^{31}P NMR spectra show characteristic sharp singlet signals ranging from 32 to 47 ppm. Bis(phosphane) cationic complexes (**5, 6** and **9**, **10**) show the highest frequency signals while the substitution of chloride with azolate does not introduce large shifts in CH_3OH solutions. In all ^1H NMR spectra the acidic protons exchange and are not visible. The ESI-MS characterization in methanol solutions shows mainly cationic fragments. A propensity to dissociation in the micromolar concentrations with formation of the bis-phosphine cationic compounds was detected in different grades for all the compounds. Anionic fragments due to azolate anions were obtained for compounds **13** - **17**.

X-ray crystal structure determination

Single crystals of the complexes 4,5– dichloroimidazolato–N)–(triphenylphosphine)–gold(I), **11**, and 4,5–dicyanoimidazolato–N)–(triphenylphosphine)–gold(I), **15**, were grown by slow diffusion of hexane into a solution of dichloromethane for **11**, and by layering hexane on a tetrahydrofuran solution for **15**. The main results of the crystal structure determinations are reported in Table 2. Table 3 lists some selected bond distances and angles of the two complexes. Figure 1 shows, in the form of ORTEP diagrams, the molecular structures of the complexes **11** and **15**, together with the selected numbering scheme. The solid–state structures of the two compounds are similar to each other; this is confirmed by a superimposition made by means of the flexible mode option of the Molecule Overlay routine in Mercury [13] (RMSD of 0.170 Å).

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In both complexes the trigonal pyramidal $–PPh_3$ moiety has a propeller–like arrangement. The P–C distances involving the C_{ipso} atoms (C4, C10, C16) in **11** and **15** are similar and are also shorter than the average (1.815 Å) found for 715 di–coordinated gold complexes showing the $–PPh_3$ residue reported in the CCDC structural database[14] (3078 observations). The mean planes encompassing the C4, C10 and C16 rings make with the mean plane of the 4,5-dichloroimidazole (or 4,5-dicyanoimidazole) ligand dihedral angles of 68.3 (C4), 82.8 (C10) and 41.6° (C16) in **11** and 42.8 (C4), 84.2 (C10) and 42.9° (C16) in **15**.

The five–membered rings of the 4,5-dichloroimidazole and 4,5-dicyanoimidazole ligands are almost perfectly planar and regular. In both complexes, the gold and phosphorous atoms lie off the mean plane of the five–membered ring by the same side; the deviation is smaller in **11**, with P and Au off by 0.09 and 0.24 Å, respectively, and greater in **15**, where the corresponding values are 0.22 and 0.59 Å. The departure is due to the deviation of the N–Au–P angle 171.30(8) ° in **11** and 172.99(9)° in **15** from the ideal value of 180°. A search in the CCDC structural database[14] indicates that the values of the angle found for **11** and **15** locate them in the lower quartile of the known range (mean value for 230 gold mononuclear di–coordinated complexes with P, N donor set is 175.2°). However, the deviation is not due to nonbonding or 'aurophilic' interactions. In fact, an inspection of the packing diagrams of the two complexes does not reveal either canonical hydrogen bonds, or intermolecular contacts shorter than 2.6 Å.

The five–membered rings of the dichloroimidazole and dicyanoimidazole ligands show some little differences (Table 3). In **11**, the bond lengths are quite similar (mean 1.349 Å, longest 1.363 Å), while there is a greater diversity in **15** (mean 1.356 Å, longest 1.380 Å). This suggests a somewhat higher degree of electron delocalization in **11** than in **15**. A further search in the CCDC database reveals that the dichloroimidazole and dicyanoimidazole ligands are not particularly common. [15, 16] By comparing our data with existing ones, it turned out that the bond distances of the 4,5-dicyanoimidazole ligand of **15** agree nicely with the mean values of reported structures, in particular with those found for the free 4,5-dicyanoimidazolate(1–) ligands in tris(2,2'–bipyridine)–cobalt(II) bis(4,5–dicyanoimidazolate) tetrahydrate [16l] and bis(2,2'–bipyridyl)–(4,5–dicyanoimidazolato)–copper(II) 4,5–dicyanoimidazolate 4,5–dicyanoimidazole [16c]. The agreement is worse for the bond distances of the dichloroimidazole ligand of **11**, where the better match is with 4,5–dichloro–1–methyl–1H–imidazole [15j]; curiously, the bond lengths found in **11** and **15** fit better with those reported for the ligands unbound to a metal center.

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In the gold coordination environment, the Au1–P1 / Au1–N1 bond lengths of **11** and **15** are 2.2328(9) / 2.040(3) Å and 2.2317(9) / 2.067(3) Å, respectively; hence, a small difference is found only for the Au–N bond length. The mean values for the Au–P / Au–N distances in 135 [14] mononuclear di–coordinated gold complexes showing the P1N1 donor set are 2.236 / 2.066 Å, respectively. Thus, Au–P and Au–N bond lengths of **11** and **15** are shorter than the reported averages and quite close to the limit for the lowest quartile of known ranges (Au–P 2.230 Å, Au–N 2.042 Å), except for the Au–N bond of **15**. The same results were obtained by restraining the search, within the previous set of 135 structures, to gold complexes where the nitrogen donor belongs to an imidazole ring. In this reduced set [17] (no 4,5-dichloroimidazole or 4,5-dicyanoimidazole ligands are present) the mean Au–P and Au–N distances are 2.236 and 2.049 Å; the molecules most closely resembling our compounds (referring to the P–Au–I fragment; I = imidazole ring, all bond lengths plus bond angles within the ring) are 1–(tricyclohexylphosphine–gold(I))–2–isopropyl–imidazole [17a], (tributylphosphine)–(9H–purin–9–ato)–gold(I) [17i] and (2–methylbenzimidazolyl–κN)– (triphenylphosphine–κP)–gold(I) [17l]. Those showing instead the most resembling distances about Au are (1,3–dimethyl–2,6–dioxo–2,3,6,7–tetrahydro–1H–purine–7–yl)–(dimethylphenylphosphine)–gold(I) [17e] (with **15**; Au–P / Au–N of 2.232 / 2.071 Å), (triphenylphosphine)–(9H–purin–9–ato)–gold(I)[17h] and, again (tributylphosphine)–(9H–purin–9–ato)–gold(I)[17i] (with **11**; Au–P / Au–N of 2.236 / 2.042 Å[17h] and 2.233 / 2.046 Å [17i]).

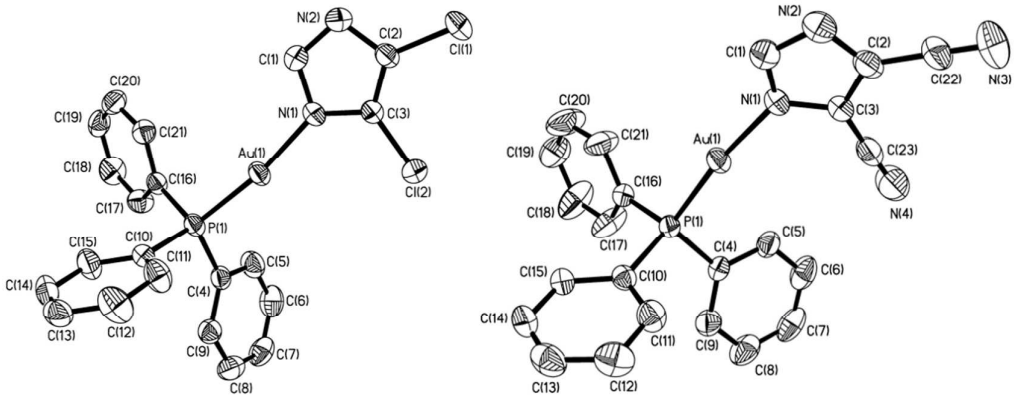

Figure 1. ORTEP [18] views of complexes 11 (left) and 15 (right), showing also the numbering scheme used. Thermal ellipsoids have been drawn at the 40% probability level; hydrogen atoms have been omitted.

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Tabella 2. Crystal data for compound **11** and **15**.

	11	**15**
Empirical formula	$C_{21}H_{16}N_2PCl_2Au$	$C_{23}H_{16}N_4PAu$
Formula weight	595.19	576.33
Temperature/K	295.9(1)	277.6(1)
Crystal system	triclinic	orthorhombic
Space group	$P-1$	$P\,2_1\,2_1\,2_1$
a / Å	9.2480(2)	6.6863(1)
b / Å	10.0825(2)	12.1176(2)
c / Å	12.0645(2)	26.3295(4)
α / °	111.258(2)	90.00
β / °	99.382(1)	90.00
γ / °	98.215(2)	90.00
Volume /Å^3	1009.10(3)	2133.28(6)
Z	2	4
ρ_{calc} / Mg/m^3	1.959	1.794
μ / mm^{-1}	7.642	6.987
F(000)	568.0	1104.0
Crystal size / mm	0.50 × 0.30 × 0.20	0.10 × 0.06 × 0.05
Reflections collected	16888	32150
Independent reflections /	5453 / 0.0324	5182 / 0.0360
Data / restraints /	5453 / 0 / 244	5182 / 238 / 262
Goodness–of–fit [a] on F^2	1.032	1.071
Final R indexes [$I > 2\sigma(I)$]	R_1 [b] = 0.0276, wR_2 [c] = 0.0626	R_1 [b] = 0.0262, wR_2 [c] = 0.0522
Largest diff. peak / hole / e	1.83/–1.29	0.63/–0.77

[a] Goodness–of–fit = [Σ (w (Fo2 – Fc2)2] / (Nobsevns – Nparams)]1/2, based on all data;

[b] R1 = Σ (|Fo| – |Fc|) / Σ |Fo|;

[c] wR2 = [Σ[w (Fo2 – Fc2)2] / Σ[w (Fo2)2]]1/2.

Table 3. Selected Bond Lengths (Å) and angles (deg) for the complexes **11** and **15.**

	11	**15**

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Au1–P1	2.2328(9)	2.2317(9)
Au1–N1	2.040(3)	2.067(3)
N1–C1	1.363(5)	1.332(5)
C1–N2	1.322(5)	1.337(5)
N2–C2	1.352(5)	1.371(6)
C2–C3	1.348(5)	1.380(5)
N1–C3	1.358(4)	1.361(5)
C2–Cl1	1.719(3)	
C3–Cl2	1.705(3)	
C2–C22		1.419(6)
C3–C23		1.412(6)
P1–C4	1.811(4)	1.804(4)
P1–C10	1.807(3)	1.807(4)
P1–C16	1.810(3)	1.809(4)
N1–Au1–P1	171.30(8)	172.99(9)
Au1–P1–C4	114.2(1)	110.4(1)
Au1–P1–C10	113.2(1)	112.2(1)
Au1–P1–C16	110.1(1)	114.5(1)
Au1–N1– C1	120.5(2)	130.8(3)
Au1–N1– C3	135.4(2)	122.7(3)
N1–C1–N2	113.9(3)	114.1(4)
N1–C3–C2	107.8(3)	106.5(4)
C1–N2–C2	103.3(3)	103.2(4)
C3–C2–N2	111.1(3)	110.2(4)
C1–N1–C3	103.9(3)	106.0(3)
C2–C22–N3		179.0(7)
C3–C23–N4		178.5(5)

(a)

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[a] The precision of the data listed in the above table was as follow : four decimal digits for Au–P distances; three decimal digits for other distances; two decimal digits for the bond angle involving Au; one decimal digit for other bond angles.

In vitro inhibition studies

Compounds **1 - 17** have been tested as DHFR inhibitors, and to evaluate their action the tests were led also with methotrexate, with PPh_3PAuCl (PPh_3Au or related moieties are present in most of the tested compounds) and with Auranofin representing a well known gold drug, whose inhibitory action has been well established.[19] Moreover the bare azoles were tested too, to evaluate the action of the gold on the inhibitory effect. The screening of the target compounds was carried out spectroscopically using an enzyme inhibitory assay against *E. coli* DHFR, in which the enzyme mediated the conversion of dihydrofolate to tetrahydrofolate, in the presence of NADPH, observing the inhibition of DHFR in the presence of gold(I) compounds. The inhibitory assay was conducted at different substrate concentrations as explained in the experimental section to assess the inhibitory mechanism and measure the K_i values. As reported in Table 4 the Ki values of **1** - **17** gold(I) compounds were in the micromolar range. The most potent compounds were **1** (1.16 μM), **4** (1.10 μM), **13** (1.05 μM) and **15** (1.06 μM), whose K_is are very similar to the value of $ClAuPPh_3$ (1.21 μM). These results seem to indicate that the primary role in the interaction of gold(I) complexes with DHFR active site residues is played by the triphenylphosphane gold(I) moiety. On the other hand, both pyrazole and imidazole groups did not dramatically alter the inhibition constants, suggesting a possible involvement of azolates in enzyme-gold(I) compound interactions. Furthermore, the introduction of a carboxyl group in one of the phenyl rings did not strongly affect the inhibition constants of compounds **3** and **4** with respect to $ClAuPPh_3$ but it can be pointed out that the *para*-substitution is better tolerated than the one in *ortho*. The *para*-carboxyl group has been detrimental to the DHFR inhibitory activity of compound **16** that showed a K_i 20-fold higher than the non-carboxylated gold(I) compound **15**. Interestingly most of the gold(I) containing complexes displayed a stronger inhibitory activity than the bare azoles. Auranofin, with a K_i value of 0,0631 $\pm$ 0,0087 μM, resulted to be the most potent gold(I) based inhibitor of DHFR of this study.

Table 4. Ki values for the investigated compounds. The methotrexate Ki value was obtained in this work with same experimental condition.

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COMPOUND	Label	Ki (μM)± S. D.
3,5-$Pz(CF_3)_2AuPPh_3$	**1**	1.16 ± 0.71
3,5-$Pz(NO_2)_2AuPPh_3$	**2**	2.57 ± 0.83
(4-COOH-Ph)Ph_2PAuCl	**3**	2.25 ± 0.62
(2-COOH-Ph)Ph_2PAuCl	**4**	1.10 ± 0.3
[(4-COOH-Ph)$Ph_2P]_2AuCl$	**5**	27.84 ± 12.12
[(2-COOH-Ph)$Ph_2P]_2AuCl$	**6**	2.066 ± 0.8
(4-$COOEt_3NH_4^+$-Ph)Ph_2PAuCl	**7**	19.66 ± 1.53
(2-$COOEt_3NH_4^+$-Ph)Ph_2PAuCl	**8**	N . D.*
[(4-$COOEt_3NH_4^+$-Ph)$Ph_2P]_2AuCl$	**9**	8.97 ± 2.78
[(2-$COOEt_3NH_4^+$-Ph)$Ph_2P]_2AuCl$	**10**	8.67 ± 1.76
4,5-$Im(Cl)_2N$- $AuPPh_3$	**11**	8.63 ± 2.24
1-Bz-4,5-$Im(Cl)_2$-C-$AuPPh_3$	**12**	19.66 ± 1.53
4,5-$Im(Cl)_2$-N-$AuPPh_2$(4-COOH-Ph)	**13**	1.05 ± 0.61
4,5-$Im(Cl)_2$-N-$AuPPh_2$(2-COOH-Ph)	**14**	10.4 ± 1.41
4,5-$Im(CN)_2N$- $AuPPh_3$	**15**	1.06 ± 1.11
4,5-$Im(CN)_2$-N-$AuPPh_2$(4-COOH-Ph)	**16**	20.42 ± 2.98
4,5-$Im(CN)_2$-N-$AuPPh_2$(2-COOH-Ph)	**17**	9.5 ± 0.8
3,5-$Pz(CF_3)_2H$		4.5 ± 2.7
3,5-$Pz(NO_2)_2Na$		8.8 ± 2.9
4,5-$Im(Cl)_2H$		No inhibition
4,5-$Im(CN)_2H$		24.9 ± 11.3
PPh_3		6.16 ± 0.77
(4-COOH-Ph)Ph_2P		No inhibition
(2-COOH-Ph)Ph_2P		No inhibition
Methotrexate		0.00078 ± 0.00015
Auranofin		0.0631 ± 0.0087
$ClAuPPh_3$		1.214 ± 0.20

*Compound 8 decomposes during the inhibitory test and the Ki was not determined.

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Stability study in solution

The target of this study was to determine the stability of the buffered solutions (pH = 7.3) of our compounds, used for the enzymatic inhibition assays. The UV-visible absorption spectroscopy represents a sensitive and practical technique to monitor this kind of behaviours in presence of buffers.[20] The gold(I) compounds tested in this study showed a medium / good solubility in a 75 % mixture of ethanol / Hepes 20 mM, pH 7.3. The solutions obtained from the samples, which appeared clear that at a concentration of 1 mg / mL, were considered suitable for spectrophotometric analysis. The electronic spectra in the UV-VIS region were recorded at different time intervals in the range of 7 - 9 hours, using both the stock solutions at a concentration of 1 mg/mL in 75% EtOH / Hepes, and afterwards 1 : 10 dilution in Hepes buffer, in order to observe the structural changes induced by both a hydrophilic environment and a dilution. The stock solution spectra (not reported) do not show any change in an interval of 24 hours of time, while spectral changes upon dilution have been observed for most of the compounds under study. However, some positive effects on stability have been observed in the presence of carboxyl substitution in the phenyl group, as shown in Figure 2 where the spectral changes upon dilution of compounds **11** and **14** are shown at different times. The most stable gold(I) complexes were compound **12** and Auranofin for which no changes were observed for the first 3 - 7 hours after dilution.

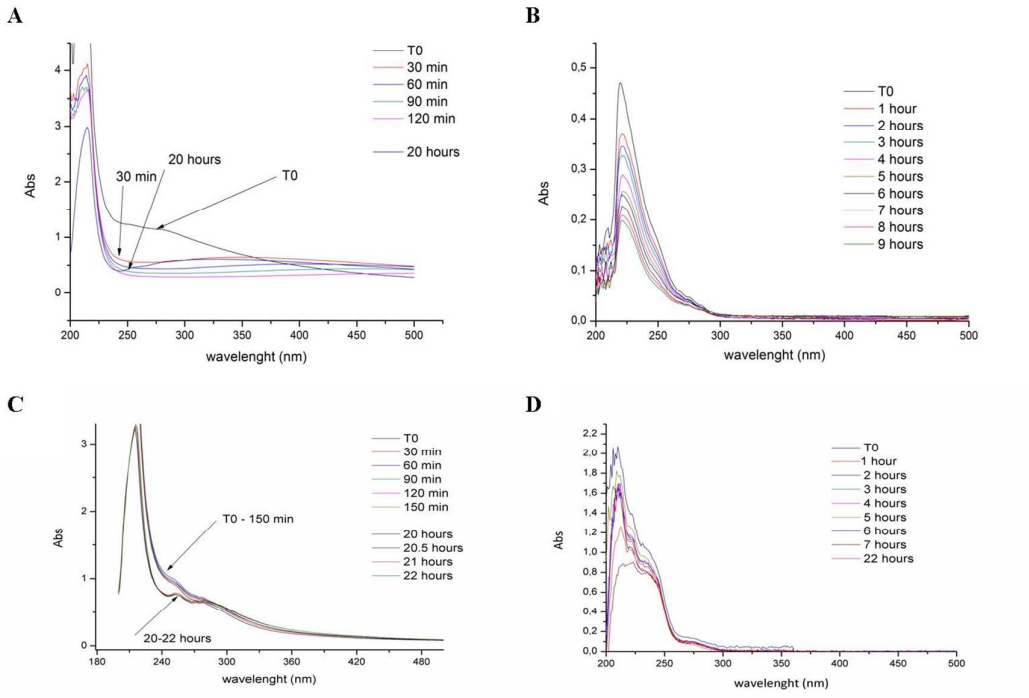

Figure 2: UV.VIS spectra of compounds **11** (A), **14** (B), **12** (C) and Auranofin (**D**) recorded at different times in a 75% ethanol / Hepes solution after dilution to 0.1 mg/ml.

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The stability study was also performed by ESI-MS spectrometry for some compounds, such as **2** – **3** - **5** - **11** – **14** - **15** and Auranofin, by using the same experimental conditions described in the experimental part. The fresh 1 mg / mL 75% ethanol/Hepes stock solutions (where Hepes is 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethansulphonic acid) were injected in the instrument. In general optimal ionic currents were achieved, and the peaks observed in these spectra did not significantly differ from those recorded in MeOH and discussed in the experimental part, , except for the peaks relative abundance. In the ESI(+) field spectra in addition to the samples peaks, , the signals at 277 m/z and trace of a peak at 515 m/z were attributed to Hepes and K^+ and to 2 Hepes + K^+, respectively, in addition to the peaks due to bis-phosphanegold cation (721 m/z and 809 m/z for the $(Ph_3P)_2Au^+$, in **2**, **11**, **15** and $(COOH\text{-}Ph\text{-}Ph_2P)_2Au^+$ for **5** and **14** respectively) and relative molecular ion or peaks related to it. These latter ones are present but with minor intensities, likely for the larger tendency to ionize of Hepes if compared to neutral gold(I) compounds. In the negative field, ions due to the negative parent ion of Hepes at 237 m/z in addition to the respective azolate m/z ions (for **2**, **11** and **15**) or the carboxylate molecular ion for **14** were recorded. For Auranofin, in the positive field ionization, Hepes stock solution's spectrum shows peaks due to Et_3PAu^+ and Et_3PH^+. In no case species due to the interaction of Hepes, or part of it, with gold compounds were revealed in these spectra. Upon dilution, the peaks due to the gold(I) compounds diminish their intensity and in some case, (such as in compound **15**) they become trace. By recording the spectra after 24 hours on the diluted solution stored on the darkness, we could observe the very low intensity of the peaks due to the gold compounds (in compounds **2**, **14** and **15** and Auranofin they completely disappear) and the appearance of peaks due to the fragmentation of Hepes such as 106.9 m/z due to piperazine moiety + Na^+ adduct, 236 m/z due to Hepes without HSO_3 + $2K^+$ in addition to numerous peaks due to light species in the range 100-250 m/z were detected.

Fluorimetric studies

The quenching of tryptophan fluorescence by gold(I) complexes has been used to analyze the ability of the apo-DHFR to interact with the synthesized gold(I) compounds. *E. coli* DHFR contains five Trp residues at positions 22, 30, 47, 74, and 133. Trp22 is located in the active site and it is involved in substrate binding stabilization through hydrogen bonding. We have tested compounds **12** and **15** to compare the quenching behaviour of two imidazolyl complexes bound to Au atom through C or N atom, respectively. The excitation wavelength was 280 nm, and maximum emission

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wavelength was 350 nm. By adding gold(I) compounds a decrease of fluorescence intensity was observed, without any shift in maximum wavelength, indicating that the presence of the gold complexes did not induce any change in the environment around Trp residues, as shown in Figure 3. Fluorescence data have been fitted by using the modified Stern-Volmer equation:

$$\log\left[\frac{F_0 - F}{F}\right] = \log K_a + n\log[Q]$$

by which it is possible to obtain the static quenching constant, also known as the binding constant, K_a, and number of binding sites (n) between the protein and metal complexes. For compound **12** we obtained a $K_a = 8.32 \times 10^4$ M^{-1} and n = 0.8, while for compound **15** we obtained $K_a = 9.5 \times 10^7$ M^{-1} and n = 1.7. The error in the K_a values was around 10%.

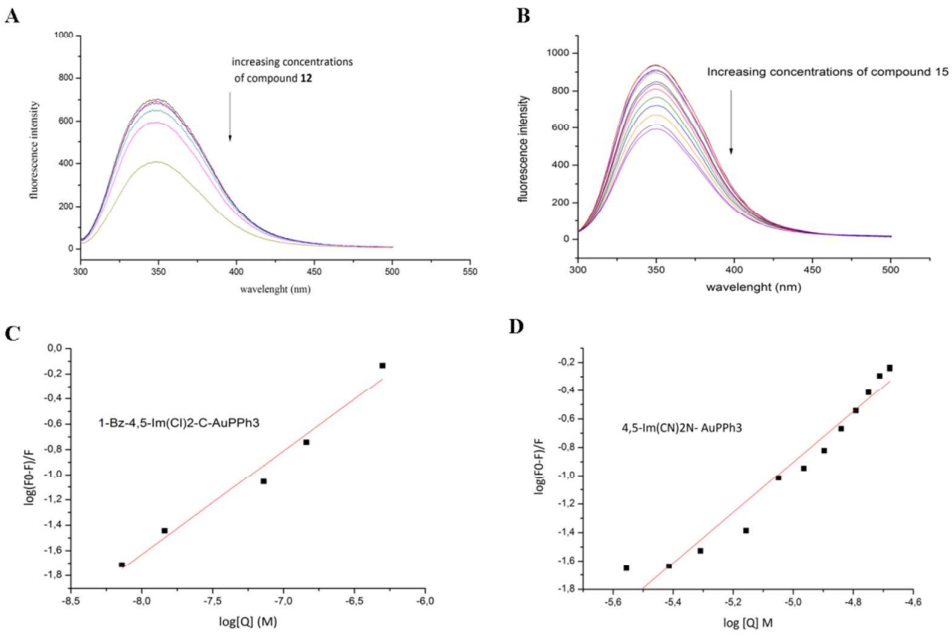

Figure 3: Fluorescence spectra of DHFR at increasing concentrations of compound **12** (A), and **15** (B) and relative derived Stern-Volmer plot (C and D).

DTNB assay

The DTNB test allows the titration of free sulfidryl groups, as in example, of cysteines in proteins.[21] Hence, this assay can rule out the interaction of the gold compounds with the cysteines. Moreover, the DHFR enzyme possesses two cysteines. It is proved that these two cysteines do not participate to the catalytic activity; in fact the double mutant C85S/C152E is active as well as the

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wild type.[22] In the present work this test was used to evaluate the interaction of a gold(I) compound with the cysteines of the enzyme. We chosen compound **3** as a model and we observed after incubation of the enzyme the presence of the two free sulfidryl groups per molecule of DHFR (both native and urea-denatured) according to the calibration curves obtained with DTT in absence ($y = 0.0117x + 0.0184$, $R^2 = 0.9704$) and in presence of 8 M urea ($y = 0.0077x + 0.0085$, $R^2 = 0.9927$). Cysteines titration of DHFR after incubation with compound **3** for 15 minutes showed a ratio between free sulfydryl groups and DHFR approximately equal to 2 : 1, as presented in Figure 4.

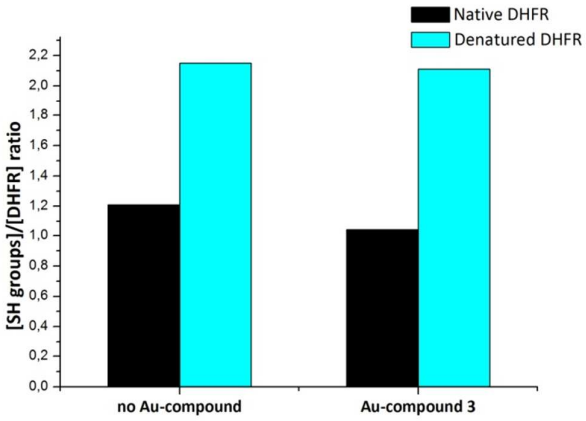

Figure 4: Ratio between sulfidryl groups and DHFR in absence and in presence of compound **3** determined both in the native and in the urea-denatured protein

Because one molecule of DHFR contains two Cys residues we could assess that compound **3** did not covalently modify the two cysteines. These results induced us to exclude the interaction of this gold(I) compound with the *E. coli* DHFR cysteines, even though a meaningful inhibition activity was appreciated (Ki = 2.25 μM).

Discussion and conclusions

In this paper, for the first time, gold(I) compounds have been studied as *E. Coli* DHFR inhibitors by *in vitro* studies. The DHFR Ki values of the herein tested compounds are of micromolar order, in contrast with Ki nanomolar value for methotrexate (0.78 nM) and K_i values for compounds **1**, **2** and **11** (15.8 nM, 3.5 nM and 7.14 nM, respectively) obtained for the inhibition of Thioredoxine Reductase enzyme (TrR).[10a] As a consequence, it is clear that this panel of the gold(I) compounds as DHFR inhibitors resulted to be active but less powerful than methotrexate and auranofin.

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However, from this study we can draft some important conclusions. By comparing the Ki values reported in table 4, it is important to note that the presence of the gold centre activates the inhibitory effect of some ligands such as the 4-benzoic-diphenylphosphinephosphane acid, 2-benzoic-diphenylphosphinephosphane acid or 4,5-$ImCl_2H$ for whose non inhibitory effects were detected; in fact, the corresponding gold compounds such as **3**, **4** and **11**, show inhibitory constants ranging from 2.25, 1.10 and 8.63 μM, respectively. The Kis data obtained for bisphosphane complexes, **5**, **6** and **9**, **10**, do not allow the outline of a structure /activity relationship, but as an overall result we can assess that the increased polarity of these ionic complexes hampers their interaction with the enzyme.[23] Compound **8** was not stable enough in the analysis media to determine the Ki. With the aim to evaluate the stability of the compound on the buffer solutions, stock solutions of the gold(I) compounds were monitored spectroscopically and spectrometrically. Most of them resulted to be stable in Hepes buffered solution for the last of the inhibition test, while they underwent dismodifications after dilution 1 : 10 in a time gap of 7 - 9 hours. The most stable compounds in this buffer after dilution are compounds **12**, and it should be noted that compound **12** is the only compound having a C-Au-P chemical environment. Nevertheless, compound **12** has a high Ki value (Ki = 19.66 μM) and a lower binding constant (K_a = $8.32 \times 10^{4}\ M^{-1}$) if compared to the analog compound **15**, having a Ki 20 times lower (Ki = 1 μM) and a K_a equal to $9.5 \times 10^{7}\ M^{-1}$. Moreover, compound **12** was also found to possess a low anticancer activity on many panels of tumoral cells.[10a] A better understanding of the Ki values should consider that, so far, the favourite sites of binding in ezymes for Au(I) moieties are the selenide groups of seleno dependent residues, the thiol groups of cysteines or the nitrogen atoms of histidines. The structure of DHFR does not contain selenide residues at all, it does not contain cysteines in the active site and the deprotonation of the only two cysteines present on the DHFR (C85 and C152) do not affect the activity of the enzyme, as it results from the fact that the Cys-free double mutant (C85S/C152E) remains active as the wild type after oxidation of cysteines.[22] Then, there are other residues in the active site that are mainly involved in substrate/cofactor proper orientation, but there are not Histidines in the active site.[23] Our kinetic experiments have been performed and analyzed under steady-state conditions for all gold(I) compounds which are thus considered as reversible inhibitors. The reaction rate dependence both on substrate and inhibitor concentration allowed us to use the Dixon plot to derive our Ki values. As a consequence, a reversible mechanism of action should consider the inhibition of DHFR by integral azolate gold(I) phosphane compounds. In conclusions, our results suggest that Gold(I) azolate phosphane

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compounds are able to inhibit DHFR despite the fact that no Cys or His or Sec residues are in the active site. If we consider compound **11** and we compare the Ki values for the inhibition of Thioredoxine Reductase enzyme (Ki = 7.14 nM)[10a] and that of the inhibition of *E. Coli* DHFR (Ki = 8.63 μM), we can discuss the 3 orders of magnitude difference of the Ki values could be an effect of the absence of selenocysteines in DHFR and of the lack of influence of the cysteines on the catalytic activity of this latter, then another less specific mechanism of action might be operative. These are our new findings with respect to the known mechanisms of action proposed for Gold(I) compounds. Among the gold compounds tested in this work, Auranofin resulted to be the most active complex with a Ki = 63 nM to which more detailed studies will be addressed.

Experimental Section.

Synthesis. Elemental analyses (C, H, N, S) were performed in-house with a Fisons Instruments 1108 CHNS-O Elemental Analyser. Melting points were taken on an SMP3 Stuart Scientific Instrument. IR spectra were recorded from 4000 to 600 cm^{-1} with a Perkin-Elmer SPECTRUM ONE System FT-IR instrument. IR annotations used: br = broad, m = medium, s = strong, sh = shoulder, vs = very strong, w = weak and vw = very weak. ^{1}H and ^{31}P NMR spectra were recorded on an Oxford-400 Varian spectrometer (400.4 MHz for ^{1}H and 162.1 MHz for ^{31}P). Chemical shifts, in ppm, for ^{1}H NMR spectra are relative to internal Me_4Si. ^{31}P NMR chemical shifts were referenced to a 85% H_3PO_4 standard. The ^{31}P NMR spectroscopic data were accumulated with ^{1}H decoupling. NMR annotations used: br = broad, d = doublet, dd = double doublet, t = triplet, m = multiplet, s = singlet. Electrospray mass spectra (ESI-MS) were obtained in positive- or negative-ion mode on a Series 1100 MSD detector HP spectrometer, using an acetonitrile or methanol mobile phase. The compounds were added to reagent grade acetonitrile to give solutions of approximate concentration 0.1 mM. These solutions were injected (1 μl) into the spectrometer via a HPLC HP 1090 Series II fitted with an auto-sampler. The pump delivered the solutions to the mass spectrometer source at a flow rate of 300 μl min^{-1}, and nitrogen was employed both as a drying and nebulising gas. Capillary voltages were typically 4000 V and 3500 V for the positive- and negative-ion mode, respectively. Confirmation of all major species in this ESI-MS study was aided by comparison of the observed and predicted isotope distribution patterns, the latter calculated using the IsoPro 3.0 computer program. The used solvents were HPLC grade and they were used as purchased, unless water and oxygen sensitive reactions were led. In this last case anhydrous and

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radicals free THF was obtained by treating the solvent with Na / acetophenone under N_2 atmosphere.

Materials. 2-benzoic acid of diphenylphosphine, 4-benzoic acid of diphenylphosphine and other chemicals were purchased and used without further purification. The complex Ph_3PAuCl was synthesized from tetrachloridegold(III) acid and a double molar amount of PPh_3 in ethyl alcohol as previously reported.[24] Solid Me_2SAuCl was synthesized by reducing $HAuCl_4$ with an excess of Me_2S and further washing with methanol. Melting Points were not determined because most of the compounds decompose at low temperature and the meltings were not clearly observed. Compounds **1**, **2**, **11** and **12** were synthesized as previously reported by some of us.[10]

Synthesis of [(4-benzoic acid-diphenylphosphane)-gold(I)chloride], 3. Solid C_2H_6SAuCl (0.2002 g, 0.6797 mmol) was dissolved in 15 ml of $CHCl_3$. To this solution, 4-benzoic acid diphenylphosphine (0.2161 g, 0.6729 mmol) dissolved in 10 ml of $CHCl_3$ was added. The reaction mixture was stirred at room temperature for 3 hours and filtered over celite. The solution was evaporated to dryness. The white crude product was washed with hexane (15 mL x 2) and with diethyl ether (15 mL). The solid was dissolved in 50 mL of CH_2Cl_2 and was filtered over a celite bed, the solution was concentrated and crystallized by a mixture of CH_2Cl_2 - diethyl ether.

Yield 68%.

^{1}H NMR ($CDCl_3$, δ) : 8.14 (dd, $^3J_{H-H}$ = 8.41 Hz, $^3J_{H-P}$ = 2.00 Hz, 2H), 7.53 (m, 12H).

^{31}P NMR ($CDCl_3$, δ): 34.2.

IR (cm^{-1}): 3072.5 (w), 3056.6 (w), 2949 (w), 2813.3 (w), 2662.0 (w), 2549.9 (w), 1680.3 (vs), 1600.4 (m), 1562.6 (m), 1481 (m), 1435.2 (m), 1395 (m), 1360 (m), 1320.8 (m), 1295.8 (s), 1186.3 (m), 1161.1 (w), 1132.8 (w), 1103.5 (s), 1116.2 (m), 998.8 (m), 947.3 (br, m), 855.3 (s), 817.8 (m), 764.6 (s), 742.3 (s), 711.6 (vs), 697 (vs).

ESI(-) (CH_3OH) m/z, %: 537.0 (60) [4-COO-Ph-$Ph_2PAuCl]^-$, 1074.9 (100) [4-COO-Ph-Ph_2PAuCl + 4-COOHPh-$Ph_2PAuCl]^-$; ESI(+) (CH_3OH) m/z %: 1041.3 (100) [4-COOHPh-Ph_2PAuCl + 4-COOH$Ph_2PAu]^+$.

Elemental analysis for $C_{19}H_{15}ClO_2PAu$, calcd % : C 42.36; H 2.80. Found % : C 42.31; H 2.79.

Synthesis of [(2-benzoic acid-diphenylphosphane)-gold(I)chloride], 4. Solid C_2H_6SAuCl (0.2044 g, 0.6940 mmol) was dissolved in 15 ml of $CHCl_3$. To this solution, 2-benzoic acid-diphenylphosphane (0.2095 g, 0.6940 mmol) dissolved in 10 ml of $CHCl_3$ was added. The reaction mixture was evaporated to dryness. The white crude product was washed with hexane (15 mL x 2) and with

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diethyl ether (15 mL). The solid was dissolved in 50 ml of CH_2Cl_2 and was filtered over a celite bed, the solution was concentrated and crystallized by a mixture of CH_2Cl_2- diethyl ether.

Yield 89%.

^{1}H NMR ($CDCl_3$, δ): 8.27 (m, 1H), 7.64 (m, 1H), 7.48 (m, 11H), 6.91 (dd, $^3J_{H-H}$ = 12.80 Hz, $^3J_{H-H}$ = 12.80, 1H).

^{31}P NMR($CDCl_3$, δ): 36.95 (s).

IR (cm^{-1}) : 3446 (w), 3059.3 (w), 2971.4 (w), 2874.3 (w), 2778 (w), 2617 (w), 1961.5 (w), 1708 (m), 1682.8 (vs), 1621 (m), 1583.6 (m), 1568 (m), 1480.3 (m), 1435.6 (s), 1412.1 (m), 1330.8 (m), 1294.8 (m), 1269 (s), 1256.1 (s), 1180 (m), 1149.4 (s), 1119.8 (s), 1102.5 (vs), 1061 (m), 1027.4 (m), 997.6 (m), 967.6 (m), 917.2 (m), 889.2 (m), 801 (m), 750 (vs), 712.2 (vs).

ESI(-) (CH_3OH) m/z, %: 537.0 (100) (2-COO-Ph-Ph_2PAuCl]$^-$. ESI(+) (CH_3OH) m/z %: 809 (18) [(2-COOHPh$_2$P)$_2$Au]$^+$, 1041.2 (100) [2-COOH-Ph-Ph_2PAuCl + 2-COOH-Ph-Ph_2PAu]$^+$.

Elemental analysis for $C_{19}H_{15}ClO_2PAu$, calcd % : C 42.36; H 2.80. Found % : C 42.73; H 2.69.

Synthesis of [Bis(4-benzoic acid-diphenylphosphane)-gold(I)chloride], 5.

Solid C_2H_6SAuCl (0.100 g, 0.3390 mmol) was dissolved in 10 ml of CH_3Cl. To this solution, 4-benzoic acid diphenylphosphine (0.2080 g, 0.6780 mmol) dissolved in 10 ml of $CHCl_3$ was added dropwise. The reaction mixture was evaporated to dryness and the crude product was washed with hexane (15 mL x 2). The white solid was dissolved in methanol and filtered through a paper filter. After filtration, the solution was concentrated and layered with diethyl ether.

Yield 98%.

^{1}H NMR (CH_3OD, δ): 8.09 (d, $^3J_{H-H}$= 7.7 Hz, 4H), 7.63 (m, 4H), 7.54 (m, 20H).

^{31}P NMR(CH_3OD, δ): 43.57 (s).

IR (cm^{-1}): 3749 (w), 3419.4 (w), 3032.8 (m), 2918 (m), 2581.3 (w), 1979.4 (w), 1926.3 (w), 1716.7 (vs), 1599.2 (m), 1563.2 (m), 1482.6 (m), 1435.7 (s), 1376.7 (s), 1331.5 (s), 1309 (m), 1211.4 (s), 1174.1 (s), 1118.6 (m), 1101.5 (s), 1018.2 (m), 998.3 (m), 850 (m), 799.8 (m), 751 (s), 744.2 (s), 712.2 (m), 709 (s), 691.5 (vs).

ESI(-) (CH_3OH) m/z, %: 537.0 (88) (2-COO-Ph-Ph_2PAuCl]$^-$, 1074.9 (100) [2-COO-Ph-Ph_2PAuCl + 2-COOH-Ph-Ph_2PAuCl]$^-$;. ESI(+) (CH_3OH) m/z %: 809 (100) [(2-COOH-Ph-Ph_2P)$_2$Au]$^+$.

Elemental analysis for $C_{38}H_{30}ClO_4P_2Au$, calcd % : C 54.01; H 3.58. Found % : C 53.74; H 3.58.

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Synthesis of [Bis(2-benzoic acid-diphenylphosphane)-gold(I)chloride], 6.

Solid C_2H_6SAuCl (0.100 g; 0.339 mmol) was dissolved in 10 ml of $CHCl_3$. To this solution, 2-benzoic acid diphenylphosphane (0.208 g; 0.679 mmol) dissolved in 10 ml of $CHCl_3$ was added dropwise. The solution was dried and the crude product was washed with hexane (15 mL x 2). The white solid was dissolved in methanol and filtered off through a paper filter. After filtration, the solution was concentrated and layered with diethyl ether. 0.282 g of a microcrystalline compound was recovered.

Yield 98 %.

^{1}H NMR ($CDCl_3$, δ): 8.30 (d, $^3J_{H-H}$= 7.7 Hz, 2H), 7.715 (t, $^3J_{H-H}$ = 8.1 Hz, 4H), 7.62 (dt, $^3J_{H-H}$= 7 Hz, $^3J_{H-P}$ = 1.7 Hz, 4H), 7.52 (m, 20H), 7.00 (d, $^3J_{H-H}$ = 7.7 Hz, 2H).

^{31}P NMR ($CDCl_3$, δ): 46.99 (s).

IR (cm^{-1}) : 2923.4 (br, w), 1701.7 (vs), 1479.7 (m), 1435.3 (s), 1390.6 (m), 1233 (s), 1147 (s), 1115.4 (s), 797 (s), 745 (vs), 692 (vs).

ESI(-) (CH_3OH) m/z, %: 321 (58), 537.0 (100) (2-COO-Ph-$Ph_2PAuCl]^-$. ESI(+) (CH_3OH) m/z %: 809 (100) $[(2\text{-COOH-Ph-}Ph_2P)_2Au]^+$.

Elemental analysis for $C_{38}H_{30}ClO_4P_2Au + H_2O$, calcd % : C 52.88; H 3.73. Found % : C 52.83; H 3.68.

Synthesis of [triethylammonium][(4-benzoate-diphenylphosphane)-gold(I)chloride)], 7.

4-benzoic acid diphenylphosphine gold(I)chloride (0.030 g; 0.056 mmol) was dissolved in 10 ml of CH_3OH. To this solution 0.056 mmol of $(C_2H_5)_3N$ were added. After magnetic stirring of an hour, the purple solution was filtered over a celite bed and dried. The crude product was washed with hexane (15 mL x 2). The crystallization was performed by dissolving the solid in CH_3OH and by layering diethyl ether.

Yield 58%.

^{1}H NMR (CD_3OD, δ): 8.10 (dd, $^3J_{H-H}$ = 8.0 Hz, $^3J_{H-P}$ = 2.0 Hz, 2H), 7.59 (m, 12H), 3.20 (q, $^3J_{H-H}$ = 7.2 Hz, 6H), 1.30 (t, $^3J_{H-H}$ = 7.2 Hz, 9H).

^{31}P NMR(CD_3OD, δ): 33.81 (s).

IR (cm^{-1}) : 3370 (br, m), 3072 (vw), 3046 (vw), 2981.5 (w), 1594 (m), 1547 (m), 1479 (w), 1435 (w), 1363 (s), 1310 (sh, m), 1175 (w), 1102 (s), 1027 (w), 1012 (w), 998 (w), 868 (vw), 831 (m), 778 (m), 748 (m), 722 (m), 691 (s).

ESI(-) (CH_3OH) m/z, %: 537.0 (100) (4-COO-Ph-Ph_2PAuCl]-, 1074.9 (48) [4-COO-Ph-Ph_2PAuCl + 4-COOH-Ph-Ph_2PAuCl]-. ESI(+) (CH_3OH) m/z %: 102 (100) $[(C_2H_5)_3NH]^+$, 239 (86).

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Elemental analysis for $C_{25}H_{30}ClNO_2PAu$, calcd % : C 46.92, H 4.73, N 2.19. Found % : C 46.99; H 4.84, N 1.91.

Synthesis of [triethylammonium][(2-benzoate-diphenylphosphane)-gold(I)chloride)], 8.

2-benzoic acid diphenylphosphinegold(I)chloride (0.030 g; 0.056 mmol) was dissolved in 10 ml of CH_3OH. To this solution an excess of $(C_2H_5)_3N$ (1 mL) was added. After magnetic stirring of an hour, the solution was dried. The crude product was washed with hexane (15 mL x 2). The crystallization was performed by dissolving the solid in CH_3OH and by layering diethyl ether.

Yield 79 %.

^{1}H NMR (CD_3OD, δ): 8.19 (m, 1 H), 7.59 (t, $^3J_{H-H}$ = 8.12 Hz ,1H), 7.47 (m, 10H), 7.38 (t, $^3J_{H-H}$ = 8.12 Hz, 1H), 6.82 (dd, $^3J_{H-H}$ =12.82 ,H), 3.14 (q, $^3J_{H-H}$ = 7.21 Hz, 6H), 1.25 (t, $^3J_{H-H}$ = 7.21 Hz, 15H).

^{31}P NMR (CD_3OD, δ): 35.69 (s).

IR (cm^{-1}) : 3501 (w, br), 3052 (vw), 2980 (vw), 1614 (m), 1584 (s), 1561 (s), 1480 (m), 1436 (m), 1346 (s, br), 1279 (m), 1149 (m), 1111 (s), 1098 (s), 1063 (m), 1025 (m), 998 (w), 902 (vw), 829 (s), 749 (s), 721 (m), 709 (m), 693 (s), 604 (m).

ESI(-) (CH_3OH) m/z, % : 537.0 (100) (2-COO-Ph-$Ph_2PAuCl]^-$. ESI(+) (CH_3OH) m/z %: 102 (100) $[(C_2H_5)_3NH]^+$, 239.3 (64) $[(C_2H_5)_3N + K]^+$, 640.3 (22) [2-COOH-Ph-$Ph_2PAuCl + C_2H_5)_3NH]^+$.

Elemental analysis for $C_{25}H_{30}ClNO_2PAu$, calcd % : C 46.92, H 4.73, N 2.19. Found % : C 47.29; H 4.81, N 2.32.

Synthesis of Bis[triethylammonium][Bis(4-benzoate-diphenylphosphane)-gold(I)chloride], 9.

bis(4-benzoic acid diphenylphosphine)gold (I)chloride (0.020 g; 0.0237 mmol) was dissolved in 10 ml of CH_3OH. To this solution an excess of $(C_2H_5)_3N$ (1 mL) was added. After magnetic stirring of an hour, the solution was dried. The crude product was washed with hexane (15 mL x 2). The crystallization was performed by dissolving the solid in CH_3OH and by layering diethyl ether.

Yield 86 %.

^{1}H NMR (CD_3OD, δ) : 8.05 (d, $^3J_{H-H}$ =7.6 Hz, 4H), 7.56 (m, 24H), 3.21 (q, $^3J_{H-H}$ = 7.2 Hz, 12H), 1.31 (t, $^3J_{H-H}$ = 7.2 Hz, 18H).

^{31}P NMR (CD_3OD, δ): 44.46 (s).

IR (cm^{-1}): 3394.4 (w), 3050.5 (w), 2980.2 (w), 2940.6 (w), 2602.1 (w), 2324.8 (w) 2192.6 (w), 1980 (w), 1603.2 (sh, s), 1593.4 (s), 1544 (s), 1476 (m), 1435.7 (m), 1366.4 (vs), 1307.2 (m), 1252.6 (m), 1156.1 (m), 1093.9 (s), 1012.7 (m), 998 (s), 864.6 (s), 830.7 (vs), 776 (vs), 748.8 (vs), 720.5 (vs).

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ESI(-) (CH_3OH) m/z, %: 305 (100); 537.0 (61) (4-COO-Ph-Ph_2PAuCl]$^-$, 807 (78)[(4-COO-Ph-Ph_2P)$_2$-Au]$^-$; 1039 (23) [4-COO-Ph-Ph_2PAuCl + 4-COO-Ph-Ph_2PAu]$^-$. ESI(+) (CH_3OH) m/z %: 102 (100) $[(C_2H_5)_3NH]^+$.

Elemental analysis for $C_{50}H_{60}ClN_2O_4P_2Au$+ $3H_2O$, calcd % : C 54.52, H 6.04, N 2.54. Found % : C 54.11; H 5.74, N 2.77.

Synthesis of Bis[triethylammonium][Bis(2-benzoate-diphenylphosphane)-gold(I)chloride], 10.

Bis[(2-benzoic acid-diphenylphosphine)]gold(I)chloride (0.020 g, 0.0237mmol) was dissolved in 10 ml of CH_3OH. To this solution an excess of $(C_2H_5)_3N$ (1 mL) was added. After magnetic stirring of an hour, the solution was dried. The crude product was washed with hexane (15 mL x 2). The crystallization was performed by dissolving the solid in CH_3OH and by layering with diethyl ether. Yield 78 %.

^{1}H NMR (CD_3OD, δ): 8.24 (d, broad, $^3J_{H-H}$ =6 Hz, 2H), 7.48 (m, 24H), 6.84 (q, $^3J_{H-H}$ = 7.2 Hz, 2H), 3.08 (q, $^3J_{H-H}$ = 7.6 Hz, 12H), 1.19 (t, $^3J_{H-H}$ = 7.6 Hz, 18H).

^{31}P NMR (CD_3OD, δ): 46.57 (s).

IR (cm^{-1}) : 3055 (w), 2938.6 (w), 2848.3 (w), 2162.6 (w), 1982.4 (w), 1604 (s), 1558 (s), 1509 (m), 1480.4 (m), 1435.6 (s), 1361.3 (s), 1287.6 (sh, m), 1182 (m), 1156.8 (m), 1099 (s), 1062 (m), 1014 (m), 998 (m), 829 (s), 748.8 (s), 720.7 (vs).

ESI(-) (CH_3OH) m/z, %: 537.0 (53) (2-COO-Ph-Ph_2PAuCl]$^-$, 807 (100) [(2-COO-Ph-Ph_2P)$_2$AuCl]$^-$. ESI(+) (CH_3OH) m/z %: 102 (100) $[(C_2H_5)_3NH]^+$; 239 (43); 809 (84) [(2-COO-Ph-Ph_2P)(2-COOH-Ph-Ph_2P)AuCl]$^+$.

Elemental analysis for $C_{50}H_{60}ClN_2O_4P_2Au$ + $5H_2O$, calcd % : C 52.80, H 6.20, N 2.46. Found % : C 51.85; H 5.24, N 2.32.

Synthesis of [(4,5-dichloro-imidazolyl-1yl)-(4-benzoic acid-diphenylphosphane)-gold(I)], 13.

Solid imidazole (0.020 g, 0.146 mmol) was dissolved in 2.9 mL of a 0.1 M methanol solution of NaOH (0.292 mmol). After 30 minutes was added solid para substituted $C_{19}H_{15}O_2PAuCl$ (0.079 g; 0.146 mmol). After magnetic stirring for 2 hours, the solution was dried by vacuum. The crude product was dissolved of ethanol and filtered over a celite bed. After filtration ,the solution was dried by vacuum. The solid was dissolved in 10 mL ethanol, and treated with 1.8 ml of 0.1 M HCl. The resulting suspension was filtered off through a paper filter and dried to obtain 0.062 g. Yield 68 %.

^{1}H NMR (CD_3OD, δ) : 8.07 (dd, $^3J_{H-H}$ = 8.2 Hz, $^3J_{H-P}$ = 1.6 Hz, 2H), 7.61 (m, 12H), 7.30 (s, 1H).

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^{31}P NMR (CD_3OD, δ) : 31.91 (s).

IR (cm^{-1}): 3052.9 (br, m), 2163 (vw), 2050.1 (vw), 1980.4 (vw), 1595.3 (s), 1547 8s), 1506.8 (m), 1482.3 (m), 1436 (s), 1384 (vs), 1306.9 (m), 1265.1 (m), 1224.8 (s), 1189 (m), 1101.6 (m), 1116.6 (m), 974 (m), 926 (w), 842.9 (m), 809.8 (w), 776 (s), 725.7 (s), 713.1 (s), 689.9 (vs), 667.8 (s), 632.4 (m), 599.4 (m), 556.1 (vs).

ESI(-) (CH_3OH) m/z, %: 135 (100) $(4,5\text{-}ImCl_2)^-$, 468.7 (38) $[(4,5\text{-}ImCl_2)_2Au]^-$. ESI(+) ($CH_3OH$) m/z %: 63 (35), 413 (100); 445 (21).

Elemental analysis for $C_{22}H_{15}Cl_2N_2O_2PAu + 3H_2O$ calcd % : C 38.17, H 3.06, N 4.05. Found % : C 37.89; H 3.24, N 3.77.

Synthesis of [(4,5-dichloro-imidazolyl-1yl)-(2-benzoic acid-diphenylphosphane)-gold(I)], 14.

20 mg of $C_3H_2Cl_2N$ (0.146 mmol) were in 2.9 ml of a 0.1 M methanol solution of NaOH. After 30 minutes solid ortho substituted $C_{19}H_{15}O_2PAuCl$ (0.0786 g; 0.146 mmol) was added. After magnetic stirring for 2 hours, the suspension was dried by vacuum. The crude product was dissolved in 10 mL of ethanol and filtered over a celite bed. After filtration, the solvent was removed by vacuum, the residue washed with diethyl ether and after drying, 0.057 g of a white solid were obtained Yield 62 %.

^{1}H NMR (CD_3OD, δ): 8.14 (dd, $^3J_{H\text{-}H}$ = 8Hz, $^3J_{H\text{-}P}$ = 4Hz, 1H), 7.49 (m, 12H), 7.28 (s, 1H), 6.85 (m, 1H).

^{31}P NMR (CD_3OD, δ) : 33.09 (s).

IR (cm^{-1}) : 3053 (w), 2848.7 (w), 1979 (w), 1690.1 (m), 1568.3 (w), 1479 (w), 1435.9 (m), 1296.4 (w), 1256 (m), 1223.5 (m), 1146.9 (m), 1098.8 (s), 1026 (m), 997.9 (m), 955 (m), 876 (w), 799.6 (m), 746 (s), 710.8 (s), 665 (vs).

ESI (CH_3OH) m/z, %: 134.9 (100) $(4,5\text{-}ImCl_2)^-$, 321 (32), 468.7 (74) $[(4,5\text{-}ImCl_2)_2Au]^-$, 536.8 (15) $[2\text{-}COO\text{-}Ph\text{-}Ph_2PAuCl]^-$, 636.8 (26) $(2\text{-}COO\text{-}Ph\text{-}Ph_2PAu\text{-}4,5\text{-}ImCl_2)^-$. ESI(+) ($CH_3OH$) m/z %: 80.1 (46), 361 (32), 639 (38) $(2\text{-}COOH\text{-}Ph\text{-}Ph_2PAu\text{-}4,5\text{-}ImCl_2H)^+$, 809 (100) $[(2\text{-}COOH\text{-}Ph_2P)_2Au]^+$, 1141 (25) $[4\text{-}COOH\text{-}Ph\text{-}Ph_2PAu\text{-}4,5\text{-}ImCl_2 + 2\text{-}COOH\text{-}Ph\text{-}Ph_2PAu]^+$.

Elemental analysis for $C_{22}H_{17}Cl_2N_2NaO_2PAu + H_2O$ calcd % : C 31.75, H 2.54, N 3.37. Found % : C 32.37; H 2.17, N 3.30.

Synthesis of [(4,5-dicyano-imidazolyl-1yl)-(triphenylphosphane)-gold(I)], 15.

0.050 g of 4,5-dicyanoimidazole (0.423 mmol) were dissolved in a methanol-water mixture (8 CH_3OH : 1 H_2O). To this solution solid K_2CO_3 (0.059 g; 0.423 mmol) was added. The mixture

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reaction was stirred overnight at room temperature. The day after, 8 ml of a solution of Ph_3PAuBF_4 in THF (0.423 mmol) was added. After magnetic stirring of 3 hours, the suspension was filtered and dried. The solid was dissolved in CH_2Cl_2 and filtered. This solution was crystallized by a mixture of CH_2Cl_2- diethyl ether.

Yield 63 %.

^{1}H NMR (CD_2Cl_2, δ): 7.59 (m, 16H).

^{31}P NMR (CD_2Cl_2, δ): 32.04 (s).

IR (cm^{-1}) : 3214.4 (br, w), 3047 (w), 2228 (m), 1572 (br, m), 1499 (m), 1471 (m), 1480.5 (m), 1446 (sh, m), 1435 (vs), 1331.5 (m), 1303 (m), 1249 (m),1202 (m), 1181 (m), 1159.3 (w), 115 (m), 1103 (s), 1072 (m), 1027 (m), 998 (m), 920 (m), 878 (s), 849 (w), 754 (m), 745 (s), 712 (s), 692 (vs), 661 (vs).

ESI(-) (CH_3OH) m/z, % : 117 (86) $[4,5\text{-}ImCN_2]^-$, 431 (100) $[(4,5\text{-}ImCN_2)_2 + Au]^-$. ESI(+) (CH_3OH) m/z % : 721 (100) $[(PPh_3)_2Au]^+$, 1035 (30).

Elemental analysis for $C_{23}H_{16}N_4PAu$ calcd % : C 45.49, H 2.66, N 9.23. Found % : C 46.02; H 2.66, N 9.23.

Synthesis of [(4,5-dicyano-imidazolyl-1yl)-(4-benzoic acid-diphenylphosphane)-gold(I)], 16.

0.046 g of 4,5-dicyanoimidazole (0.389 mmol) were dissolved in a methanol-water mixture (8 CH_3OH : 1 H_2O). To this solution was added solid K_2CO_3 (0.054 g; 0.389 mmol). The mixture reaction was stirred overnight at room temperature. The day after, 8 ml of a solution of para substituted $C_{19}H_{15}O_2PAuBF_4$ (0.389 mmol) in THF was added. After magnetic stirring of 3 hours, the suspension was filtered and dried. The solid was dissolved in 10 mL of CH_3OH and layered with diethyl ether to obtain 0.080 g of crystal product.

Yield 33.5 %.

^{1}H NMR (CD_3OD, δ) : 8.01 (d, ^2J = 8 Hz, 2H), 7.53 (m, 12H), 7.40 (s, 1H).

^{31}P NMR (CD_3OD, δ) 44.61 (s).

IR (cm-1) : 3352 (br, m), 3056.6 (w), 2225.2 (m), 1980 (vw), 1750 (vw), 1591.5 (s)s, 1543.6 (s), 1481.5 (m), 1435.7 (s), 1374 (vs), 1250 (m), 1183.3 (m), 1158.3 (m), 1099.5 (s), 1026.6 (w), 1014.7 (m), 998.8 (m), 926 (w), 838 (m), 776.3 (m), 744.4 (s), 662 (vs).

ESI(-) (CH_3OH) m/z, %: 117 (100) $[4,5\text{-}ImCN_2]^-$, 234.9 (36) $[4,5\text{-}ImCN_2 + 4,5\text{-}ImCN_2H]^-$, 431 (39) $[(4,5\text{-}ImCN_2)2 + Au]^-$, 619 (40) $[4\text{-}COO\text{-}Ph\text{-}Ph_2\text{-}PAu\text{-}4,5\text{-}ImCN_2]^-$. ESI(+) ($CH_3OH$) m/z %: 242 (100), 659

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(70) [4-COOH-Ph-Ph_2PAu-4,5-ImCN_2 + K]$^+$, 809 (38) [(4-COOH-Ph-Ph_2P)$_2$Au]$^+$; 1123 (39) [4-COOH-Ph-Ph_2-PAu-4,5-ImCN_2 + 4-COOH-Ph-Ph_2PAu]$^+$.

Elemental analysis for $C_{24}H_{16}N_4O_2PAu$ calcd % : C 46.47, H 2.60, N 9.03. Found % : C 45.96; H 2.60, N 9.53.

Synthesis of [(4,5-dicyano-imidazolyl-1yl)-(2-benzoic acid-diphenylphosphane)-gold(I)], 17.

0.0854 g of 4,5-dicyanoimidazole (0.723 mmol) were dissolved in a methanol-water mixture (8 CH_3OH : 1 H_2O). To this solution was added solid K_2CO_3 (0.091 g, 0.723 mmol) and stirred overnight. 8 mL of a solution of freshly prepared ortho substituted $C_{19}H_{15}O_2PAuBF_4$ (0.723 mmol) in THF was added. After magnetic stirring of 3 hours, the suspension was filtered over a celite bed and dried under vacuum. The raw solid was washed with hexane (3 x 3 mL) and H_2O (3 x 5 mL). After a prolonged drying under vacuum 0.321 g of a white solid were recovered.

Yield 74 % .

^{1}H NMR (CD_3OD, δ) : 8.21 (m, 1H), 7.75 (m, 1H), 7.65 (t, $^3J_{H-H}$ = 8Hz, 1H), 7.26 (m, 10H), 6.92 (s, 1H).

^{31}P NMR (CD_3OD, δ) 33.27 (s).

IR cm^{-1}: 3340 (br, w), 2229 (m), 1686 (br, m), 1597 (m), 1580.6 (m), 1557 (m), 1481.5 (w), 1449 (w), 1436 (m), 1374 (s), 1301 (m), 1212 (m), 1250 (m), 1149 (m), 1116 (s), 1102 (s), 997 (m), 958 (w), 836 (m), 802 (m), 747 (s), 711 (m), 662 (vs).

ESI(-) (CH_3OH) m/z, % : 117 (84) (4,5-ImCN_2)$^-$, 235 (81) [(4,5-ImCN_2) + (4,5-ImCN_2)H]$^-$, 431 (82), 619 (100) [ImCN_2Au-(2-COO-Ph)Ph_2P]$^-$, 737 (74). ESI(+) (CH_3OH) m/z % :80 (100), 361 (59) [2-COO-Ph)Ph_2P + Na +MeOH]+, 659 (87) (2-COOH-Ph-Ph_2PAu-4,5ImCN_2 + K]$^+$, 809 (64), 809 (93) [(2-COOH-Ph-Ph_2P)$_2$Au]$^+$; 1043 (66).

Elemental analysis for $C_{24}H_{16}N_4O_2PAu$ calcd % : C 46.47, H 2.60, N 9.03. Found % : C 45.85; H 2.62, N 8.54.

LogP determination (Shaked Flask Method).

The LogP was determined according to the Shaked Flask Method[25] consisting in dissolving the analytical sample in 1-octanhol, shaking with pure water, leaving to restore the two phases and measuring the Absorbance of the octanhol phase. LogP values were calculated for Ph_3PAuCl, **1**, **2**, **3**, **7** and **9** compounds and their relative ligands. A calibration curve was built for each compound by dissolving 2 mgs of the compound in 10 mL of octanhol, and recording the UV-Vis spectrum at different concentrations.

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Crystallography

X-Ray structure determination and refinement.

Selected crystalline specimens of the complexes **11** and **15** were fastened on the top of a glass fiber and centered on the goniometer head of a four–circle kappa–goniometer Oxford Diffraction Gemini E diffractometer, equipped with a 2K × 2K EOS CCD area detector and sealed–tube Enhance (Mo) and (Cu) X–ray sources. Data collection was performed in both cases at room temperature, by means of ω–scans, using graphite–monochromated radiation in a 1024 × 1024 pixel mode and 2 × 2 pixel binning.

The diffraction intensities were corrected with respect to Lorentz and polarization effects. Empirical multi–scan absorption corrections, using equivalent reflections, were also performed with the scaling algorithm SCALE3 ABSPACK. Raw data collection, data reduction and refinalization were carried out with the CrysAlis Pro software.[26] Accurate unit cell parameters were determined by least–squares refinement of 8307 (**11**) and 11530 (**15**) reflections of strongest intensity, picked from the whole experiment.

Both structures were solved by means of the heavy–atom methods, using SHELXS[27] and refined by full–matrix least–squares methods based on F_o^2 with SHELXL–97[24] in the framework of the OLEX2[28] software. In the last cycles of refinement, all non-hydrogen atoms were refined anisotropically; hydrogen atoms were instead geometrically placed and refined as riding atoms with individual isotropic displacement parameters set to 1.2 times the U_{eq} of the pertinent parent atoms. With respect to data completeness, several crystals have been evaluated for both complexes; the best specimens for **11** and **15** did not diffract at θ values greater than 28.02 and 26.32°, respectively, affording a data completeness of 90.6 and 95.4% for **11** and **15**, respectively. Despite this limitation, however, the chemical and structural identification of the investigated species was not in question.

A few more comments have to be made about compound **11**. In the final stages of refinement, it appeared that the carbon atoms of the $–PPh_3$ moiety were affected by some degree of thermal motion. This was especially true for the atoms labelled C16 to C21 and some peaks appeared in positions compatible with alternate arrangements of C17, C18, C20 and C21. All the attempts made to model these carbon atoms as disordered over two sites (with occupancies constrained to sum to 1.0) proved unsatisfactory. Since the identification of the molecular structure was unambiguous, we left unsplitted the involved atoms and we refined them anisotropically,

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introducing DELU and SIMU restraints over all non-hydrogen atoms. The atom showing the final largest U_{eq} (0.106) was C20. Similar considerations also apply to the terminal N3, N4 atoms of the –C≡N groups. In this case, N4 had the final largest U_{eq} (0.109).

The space group suggested[26] for the structure during data collection was examined carefully, and the structure was found to solve and refine satisfactorily in the noncentrosymmetric and also enantiomorphic P 21 21 21 space group; refinement performed alternatively with the MERG2 and MERG3 instructions of SHELXL[27] allowed the calculation of the correct number of Friedel pairs to be 2129.

The supplementary crystallographic data for **11** and **15** have been deposited at the Cambridge Crystallographic Data Center in the form of .cif files, with CCDC numbers 997733 and 997734. The data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif.

Enzyme inhibition studies.

Preparation of the NADPH stock. 3.2 mg of NADPH were dissolved in 1 ml of Hepes buffer (4-(2-hydroxyethyl)pyperazine-1-ethansulphonate), pH = 7.3. A concentration between of 3 e 4 mM was obtained. A spectrophotometric determination by using buffer as background was performed after dilution to 1 : 50 with Hepes (20µl di NADPH and 980 µl of buffer); the absorbance was measured at 340 nm (for NADPH $\varepsilon_{340} = 6220\ M^{-1}\ cm^{-1}$).

Preparation of the H_2F stock. 1 mg of H_2F was dissolved in 600 µL of Hepes Buffer 50 nM and 38 µL of β-mercapto-ethanol. The blank reading was made with KOH 0.1 M. After dilution 1 : 200 in KOH 0.01 M (5 µL di H_2F and 995 µL of KOH), the absorbance was read at 283 nm (for H_2F $\varepsilon_{283} = 28000\ M^{-1}\ cm^{-1}$).

Preparation of the DHFR stock. The overexpressed and purified DHFR from *Escherichia coli* is stored at 4°C after precipitation with 90% of $(NH_4)_2SO_4$. Before any experiment, dialysis of the DHFR was performed in two steps against 10 mM potassium phosphate buffer (K_2HPO_4/KH_2PO_4) pH = 7.3, with the last step overnight. The concentration was determined by recording the Absorbance at 280 nm (for *E. coli* DHFR $\varepsilon_{280} = 31100\ M^{-1}\ cm^{-1}$).

Inhibition studies on DHFR. The activity of the DHFR was followed by recording spectra in a Shimadzu UV-2450 (UV-Vis spectrophotometer). The enzyme assays were led in the quartz cuvette by using the same experimental conditions which can be listed as : DHFR 0.93 µM, NADPH 40 µM, H_2F 2 µM – 5 µM – 10 µM. The procedure consists of : 950-971 µl of 50 mM Potassium Phosphate buffer at pH 7.3, 20 µl of NADPH, 5 µl of DHFR (stored at 30°C for minutes), and 4-20 µl of H_2F

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were mixed. To this reference solution, increased amount of gold(I) compounds solutions were added. The decreasing absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$, was recorded after the adding and expressed in U/mL by using the following equation: U/mL = $\Delta abs/\Delta t/11.8$ x dilution factor, where 11.8 is the extinction mM coefficient relative to the mixture of NADPH e H_2F simultaneously present in the mixture (ε_{340} of 11.8 M^{-1} cm^{-1}). Dixon and Lineweaver-Burk plots were adapted to determine the inhibition type, and the second plot of slopes from Dixon plot *vs* H_2F concentrations was used to calculate Ki value.

Fluorimetric analysis

The fluorimetric analysis was performed using a quartz cuvette 500 μl and a concentration of protein (DHFR) equal to 5 uM; azolate complexes of Au (I) were added in increasing amounts and at the same concentrations used to determine the enzyme's activity. Before starting with the acquisition of the spectra in the presence of inhibitors, the blank is done with the bare protein. The emission spectra of the protein are recorded at wavelengths between 250 and 500 nm using excitation wavelength characteristic of the absorption spectrum of Trp equal to 292 and 280 nm. The temperature of the instrument was always kept constant at 25 ° C. The fluorimetric data were analyzed using the theory of Stern-Volmer quenching of fluorescence.

DTNB assay. The sulfydryl content of DHFR, in absence and in presence of gold compounds, was determined by Ellman's method[21]. Before free cysteines determination in DHFR, a calibration curve has been performed by using dithiotreitole DTT ((DTT, F.W. = 154.235 g/mol) as standard. 50 mgs of dithionitrobenzoic acid (DTNB) in 1 ml of water, were centrifuged in Vortex to eliminate the excess. A stock of DTT 100 mM was prepared by dissolving 15 mg of DTT in 1 ml of water and diluted twice 1 : 10 to obtain a solution 1 mM. 6 samples of 1mM solutions with the final concentrations 0, 5, 10, 15, 20 e 30 μM were then prepared. The buffer used is 1 M tris hydroxymethyl aminomethane chloride (Tris-HCl) at pH 8. The six samples consisted of: 600 μl of DTT (from 0 to 30 μM), 25 μl of DTNB, 100 μl of Tris-HCl. The absorbance was recorded at 412 nm. To determine the amount of free cysteines in DHFR, 300 μl of 12 μM DHFR were added to the reaction mixture containing 25 μl of DTNB and 400 μl of Tris-HCl to reach a final concentration of 5 μM. Furthermore enzyme's sulfydryl groups titration was performed in presence of 50 μM of gold compound **3**. To determine the sulfydryl content of DHFR also in the denatured protein all the measurements were repeated in presence of 8 M urea.

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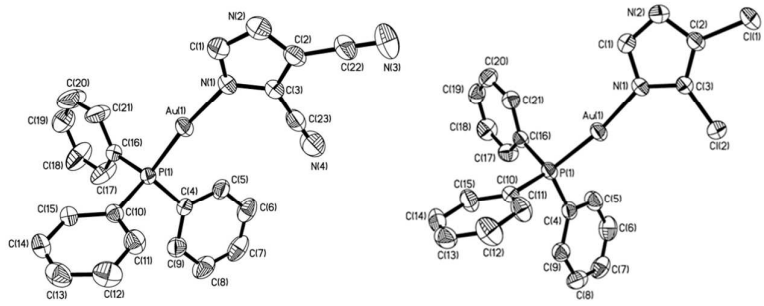
Synopsis and graphical abstract

A Study on the Inhibition of DiHydroFolateReductase (DHFR) from *Escherichia coli* by Gold(I) Phosphane Compounds. X-ray Crystal Structures of (4,5-dichloro-1H-imidazolate-1-yl)-triphenylphosphane-gold(I) and (4,5-dicyano-1H-imidazolate-1-yl)-triphenylphosphane-gold(I).

Rossana Galassi, Camille Simon Oumarou, Alfredo Burini, Alessandro Dolmella, Daniela Micozzi, Silvia Vincenzetti, Stefania Pucciarelli.*

A study on the inhibition of DeHydroFolate Reductase (DHFR) by gold(I) compounds has been performed. Auranofin, a triethylphosphane gold(I) derivative of tetraacetylthioglucose, and some azolate gold(I) triphenylphosphane complexes have been tested and Methotrexate was used as reference inhibitor. The gold(I) compounds resulted to be active in micromolar concentrations with Auranofin showing a Ki of 63 nM.



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