Full Paper

Synthesis, Antitumor, and Antibacterial Activity of Bis[4,5-diarylimidazol-2-ylidene]methane Derivatives

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Cationic [bis(1,3-diethyl-4,5-diarylimidazol-2-ylidene)]Au(I) bromide complexes have demonstrated considerable potential as new antitumor agents. In order to investigate whether the gold is crucial for the antitumor activity, the imidazole ligands were connected by a methylene bridge. Biological evaluation revealed that bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]methane compounds exhibited growth inhibition effects against mammary (MCF-7 and MDA-MB 231) and colon (HT-29) carcinoma cell lines. In comparison with gold complexes, the methylene derivatives showed drastically reduced cell growth inhibitory properties. However, the growth of bacteria was significantly inhibited by bis[1,3-diethyl-4,5-bis(4-methoxyphenyl)imidazol-2-ylidene]methane dibromide (**4**) and opens a new application of this compound type.

Keywords: Antibacterial activity / Antitumor activity / Gold–bisNHC complexes / Methylene bridged imidazoles

Received: December 30, 2011; Revised: February 7, 2012; Accepted: February 16, 2012

DOI 10.1002/ardp.201100474

Introduction

In order to overcome the disadvantages (side effects, e.g., nausea, alopecia, and development of resistance during the therapy) of the established platinum-based drugs cisplatin, carboplatin, and oxaliplatin which are widely used in the treatment of cancer, current strategies in the development of novel metallodrugs more and more focused on the use of transition metal complexes containing improved organic ligands [1, 2].

During the last years *N*-heterocyclic carbenes (NHCs) could be established as carrier ligands for metal complexes displaying high biological effects [3–6]. Especially gold–NHCs which can be seen as derivatives of auranofin and its chloro analog Et_3PAuCl (Scheme 1) recently gained much attention because of their strong antiproliferative effects [7–13]. Both phosphine complexes exhibit their antitumor activities mainly due to the inhibition of the enzyme thioredoxin reductase (TrxR) [13]. The use of NHCs as alternatives to phosphines as ligands for the soft Au(I) ion resulted in complexes with different steric and lipophilic properties [2–5, 7–13].

In our group, we intensively studied the significance of ligands derived from pharmacologically active 4,5-diarylimidazoles on the cytotoxicity of cationic Au–NHC complexes. Among them the dimeric bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]gold(I) complexes (Scheme 1) showed good growth inhibition properties. They were at least 10-fold more active than cisplatin against mammary (MCF-7 and MDA-MB 231) and colon (HT-29) carcinoma cells [7, 8].

Encouraged by these promising results and in continuation of our structure–activity relationship (SAR) studies, we extended our research to bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]methane analogs. Due to this structural modification the relevance of the gold for growth inhibitory effects can be estimated. Formally, in the cationic Au(I)–bisNHC complexes, the gold atom was exchanged by a methylene linker. All synthesized compounds were characterized by MS, NMR spectra and tested for antitumor as well as antibacterial potency *in vitro*.

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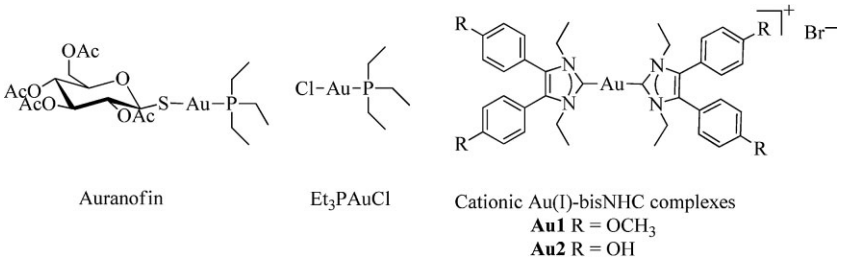
Abbreviations: 5-fluorouracil (5-FU); Luria broth (LB); *N*-heterocyclic carbenes (NHCs); phosphate buffered saline (PBS); structure–activity relationship (SAR); thioredoxin reductase (TrxR).



Scheme 1. Structures of auranofin, Et_3PAuCl, and cationic Au(I)–bisNHC complexes (**Au1** and **Au2**).

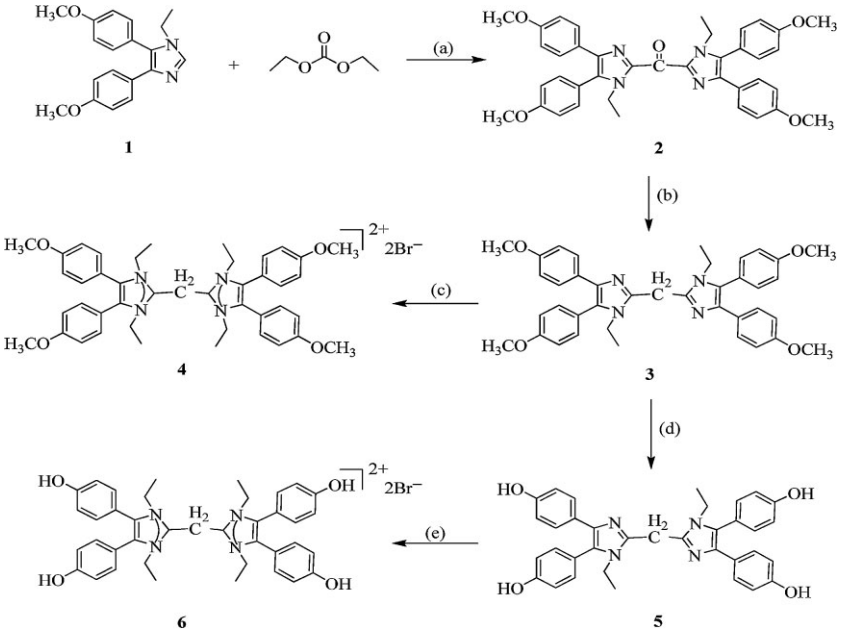
Results and discussion

Chemistry

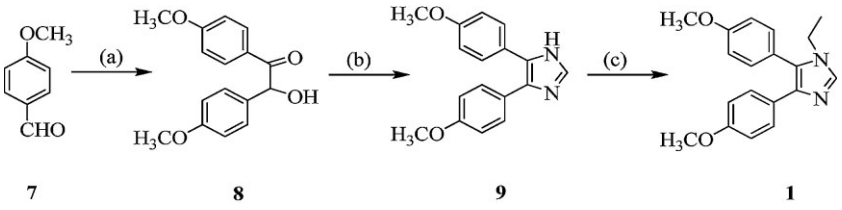
1-Ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazole (**1**), the educt for the preparation of the dimeric compounds **3**–**6** (see Scheme 2), was synthesized according to our previously published methods [6, 7]. As outlined in Scheme 3, reaction of 2 equiv. of the commercially available 4-methoxybenzaldehyde (**7**) under catalysis of thiamine hydrochloride gave 2-hydroxy-1,2-bis(4-methoxyphenyl)ethanone (**8**) which was heated in formamide to reflux for 3 h. The resulting 4,5-bis(4-methoxyphenyl)-1*H*-imidazole (**9**) was isolated and reacted with NaH and ethyl bromide in absolute THF to afford *N*-alkylation (**9**→**1**).

Deprotonation of **1** and reaction with diethyl carbonate at low temperature using a modified procedure of Rüether and co-workers [14] yielded the bis[1-ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazol-2-yl]methanone **2** (Scheme 2). Due to the relatively low acidity of the imidazole, BuLi was used as base.

For reduction to the bis[1-ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazol-2-yl]methane (**3**), the keton **2** was heated with hydrazine hydrate and potassium hydroxide [14]. The careful



Scheme 2. Synthetic routes of bis[4,5-diarylimidazol-2-ylidene]methane derivatives. Reagents and conditions: (a) *n*-BuLi, THF, −80°C to rt, 54.0%; (b) KOH, NH_2NH_2 H_2O, 120–150°C, 73.3%; (c) ethyl bromide, CH_3CN, reflux, 48–72 h, 77.6%; (d) BBr_3, CH_2Cl_2, −60°C, then warm to rt, 48 h, 65.5%; (e) ethyl bromide, CH_3CN, reflux, 48–72 h, 68.2%.



Scheme 3. Synthetic routes of 1-ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazole (**1**). Reagents and conditions: (a) thiamine hydrochloride, H_2O/ethanol 1:2, rt, 96 h, 58.8%; (b) formamide, reflux, 3 h, 75.3%; (c) 95% NaH, ethyl bromide, absolute THF, reflux, 2 h, 87.9%.

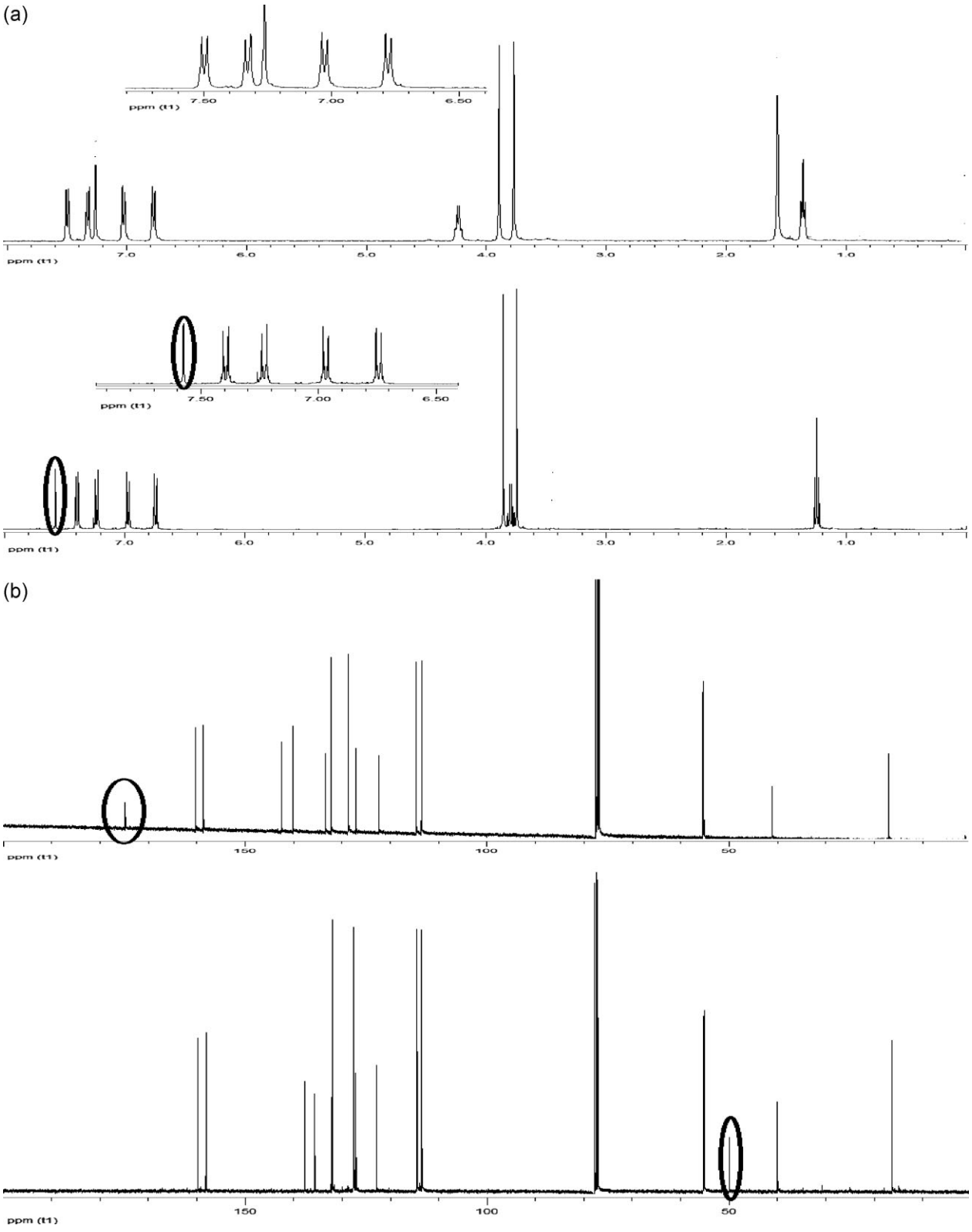


Figure 1. (a) ^{1}H NMR spectra of **2** (above) and **3** (below) (400 MHz, $CDCl_3$); (b) ^{13}C NMR spectra of **2** (above) and **3** (below) (100 MHz, $CDCl_3$).

control of the temperature is crucial to the relative success of the reaction. A yellow solid was formed at 60°C and the reaction mixture became clear and colorless when the temperature reached 116°C. The reaction was kept at 120°C for 1 h followed by heating to 150°C for another 3 h. In experiments in which the temperature was instantly raised to 150°C, the solution became dark brown and the yields were low. These observations indicate that the initial hydrazone formation has to be completed prior to the final reduction step at elevated temperature.

Following this procedure, **4** could be obtained in high yield and analytically pure after recrystallization from THF. Its structure was confirmed by NMR spectroscopy. Characteristically, the methylene protons were observed at $\delta = 7.58$ in the ^{1}H NMR spectrum of **3** (Fig. 1a). In its ^{13}C NMR spectrum the signal of the C=O group peak at $\delta = 174.7$ as determined for **2** is displaced by a CH_2 signal upfield shifted to $\delta = 49.8$ (Fig. 1b).

The bis[1-ethyl-4,5-bis(4-hydroxyphenyl)-1*H*-imidazol-2-yl]-methane (**5**) was generated from the corresponding compound **3** by ether cleavage with BBr_3. Finally, compounds **3** and **5** were further reacted with ethyl bromide in CH_3CN to yield the bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]methane derivatives **4** and **6**, respectively.

Biological activity

The growth inhibitory potency of compounds **2**–**6** as well as the antitumor drug 5-fluorouracil (5-FU) was evaluated in an established *in vitro* cytotoxicity assay using MCF-7 and MDA-MB 231 breast cancer as well as HT-29 colon carcinoma cells [15, 16]. In this test, a known number of cells were exposed to increasing concentrations (0.63–20 μM) of the compounds on 96-well tissue culture plates and incubated for a given period of time (72 h). From the concentration–activity (*%T/C*) relation the IC_{50} values were calculated (OriginPro 8) and are presented in Table 1. Additionally, the time-dependent activities of **4** on the MCF-7 cell line and of **6** on MDA-MB 231 and HT-29 cell lines are presented in Fig. 2 as examples.

Table 1. Growth inhibitory effects against MCF-7, MDA-MB 231, and HT-29 cells.

Compound	Cytotoxicity, IC_{50}[a] (μM)		
	MCF-7	MDA-MB 231	HT-29
2	>20	>20	>20
3	10.1 ± 1.2	>20	14.1 ± 4.1
4	2.6 ± 0.2	>20	7.1 ± 0.4
5	9.7 ± 0.4	11.2 ± 2.2	8.1 ± 0.6
6	5.5 ± 0.4	10.8 ± 1.0	15.5 ± 0.1
5-FU	4.7 ± 0.4	9.6 ± 0.3	7.3 ± 1.0
Au1[b]	0.17	0.54	0.43
Au2[b]	0.30	1.55	0.47

[a] The IC_{50} value was determined as the concentration causing 50% decrease in cell growth after 72 h of incubation and calculated as the mean of at least two or three independent experiments.
[b] Data from Ref. [8].

The methanone **2** was inactive in all cell lines (IC_{50} >20 μM), while its methylene analogon **3** reduced the growth of MCF-7 and HT-29 cells with $IC_{50} = 10.1$ and 14.1 μM, respectively. Interestingly, **3** was inactive against MDA-MB 231 cells (IC_{50} >20 μM). An additional *N*-ethyl group increased the growth inhibitory potency. Although **4** represents a permanent cation it was more active than 5-FU at the MCF-7 cell line ($IC_{50} = 2.6$ μM; 5-FU: $IC_{50} = 4.7$ μM) and comparably active at HT-29 cells ($IC_{50} = 7.1$ μM; 5-FU:

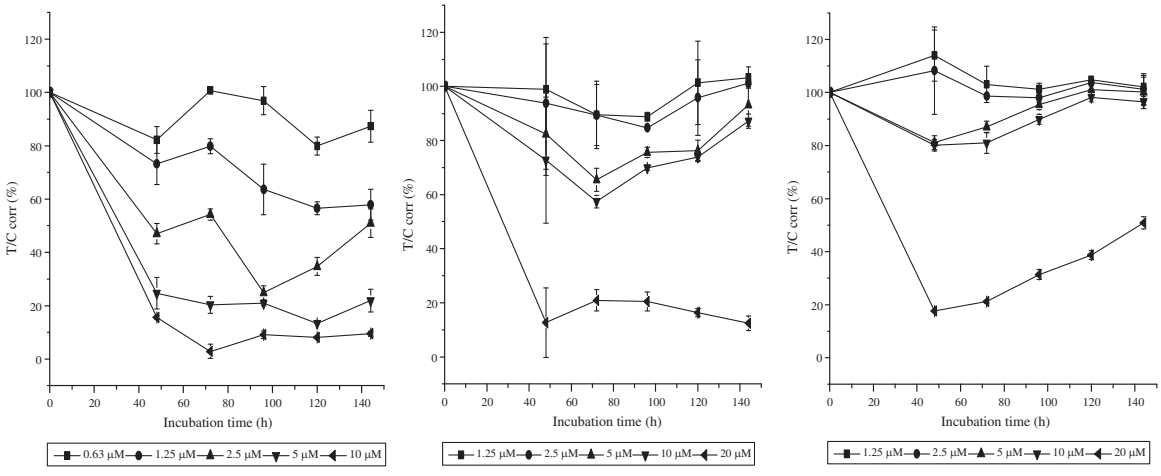


Figure 2. Time–activity curves of **4** on MCF-7 (left) cell line and **6** on MDA-MB 231 (middle) and HT-29 (right) cell lines. Error bars are hidden behind the symbols in some cases.

Table 2. Sensitivity test of compounds in inhibiting bacterial growth

Test compounds	Concentration (mM)	Diameter of the zone of inhibition (mm)			
		Ervinia amylovora **Ea 273**	***Escherichia coli*** **DH5α**	***Bacillus subtilis*** **168**	***Bacillus megaterium***
4	5	15	7	18	20
	10	17	8	30	23
6	5	5	5	5	5
	10	5	5	5	5
Au1	5	5	5	8	9
	10	5	5	10	10
$AgNO_3$	10	12	11	9	12
H_2O		5	5	5	5

IC_{50} = 7.3 μM). Again, no influence was observed on MDA-MB 231 cells (IC_{50} >20 μM).

Effects on MDA-MB 231 cells can be observed after ether cleavage resulting in compounds **5** and **6** (IC_{50} = 10.2 and 10.8 μM, respectively). At the other cell lines **6** was half as active as its methoxy derivative **4** (IC_{50} = 5.5 and 2.6 μM at MCF-7 cells; IC_{50} = 15.5 and 7.1 μM at HT-29 cells) while **5** (4-OH) and **3** (4-OCH_3) were equipotent at MCF-7 cells (IC_{50} = 9.7 and 10.1 μM). At HT-29 cells, **5** (IC_{50} = 8.1 μM) was more active than **3** (IC_{50} = 14.1 μM).

These data clearly demonstrate that the exchange of the gold center by a methylene group decreases the growth inhibitory effects. The cationic bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]Au(I) complexes Au1 (IC_{50} = 0.17 μM, MCF-7; IC_{50} = 0.54 μM, MDA-MB 231; IC_{50} = 0.43 μM, HT-29) and Au2 (IC_{50} = 0.30 μM, MCF-7; IC_{50} = 1.55 μM, MDA-MB 231; IC_{50} = 0.47 μM, HT-29) were more than 10–20-fold higher cytotoxic than the methylene derivatives **4** and **6**.

In order to exclude that the cells become resistant during the treatment, which is indicated by an increased cell growth after an initial inhibition, we investigated the time-dependent cell growth. In Fig. 2 the time–activity curves of **4** and **6** are depicted as representatives.

In both cases, the tumor cells showed marginal recuperation after a prolonged exposition to the drugs. Because the exponential cell growth is guaranteed for at least 140 h of incubation, the rise of the growth curve can be an indication of development of drug resistance. The maximal antiproliferative effects were achieved between 48 and 72 h, so we evaluated the IC_{50} value of all complexes after an incubation time of 72 h [17, 18].

Finally, because imidazolium salts and gold complexes were long term investigated as antimicrobial agents [2, 4], we evaluated the *in vitro* antimicrobial activity of target compounds **4** and **6** compared to the gold complex Au1. A modified agar diffusion test [6, 19] was used to evaluate the preliminary antibacterial activities of compounds. Indicator bacteria were grown in Luria broth (LB) at 30°C (*Ervinia amylovora* Ea273) or 37°C (*Escherichia coli* DH5α, *Bacillus subtilis* 168, and *Bacillus megaterium*). When the cell concentration of bacteria was up to 4 × 10^7 CFU/mL, 0.5 mL of bacteria suspensions were mixed with 20 mL of melting LB agar and cooled below 60°C to prepare the plates. $AgNO_3$ was used as positive control. Fifty microliters of aqueous solution with increasing compound concentrations or 50 μL of water (containing 0.1% DMSO) was loaded into the wells (diameter: 5 mm) and punched in indicator bacteria plates which were then incubated at 30°C overnight to observe the growth inhibition effects. The antimicrobial activity of the compounds was determined by measuring the diameter of the inhibiting zone around wells.

Our testing confirmed for **4** antimicrobial properties at a level comparable to silver nitrate against *E. amylovora* Ea 273, *E. coli* DH5α, *B. subtilis* 168, and *B. megaterium* (see Table 2). The maximum of antimicrobial activity was observed against the *B. subtilis* 168 and *B. megaterium*. The diameter of the inhibition zone of **4** against the *B. subtilis* 168 at 10 mM was almost threefold than that of $AgNO_3$.

Unlike compound **4**, compound **6** was inactive in our bacterial growth inhibiting assay, indicating that methoxy substituents might mediate higher potency than hydroxyl substituents against bacteria. Moreover, unlike its high antitumor activity, the gold complex Au1 was less active than compound **4**. Au1 reached nearly the activity of $AgNO_3$ against *B. subtilis* 168 and *B. megaterium*, but it was even inactive against *E. amylovora* Ea 273 and *E. coli* DH5α.

Conclusion

In conclusion, bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]-methane compounds were synthesized and tested for biological activities. They possessed growth inhibitory effects dependent on the substituents at the aromatic rings. The lower influence compared with the related gold complexes documented that the gold core seems to be responsible for the high cytotoxic activity of cationic bis[1,3-diethyl-4,5-diarylimidazol-2-ylidene]Au(I) complexes. Antibacterial tests showed

that compound **4** exhibited higher activity than $AgNO_3$ and the gold complex Au1 and open a new application of this type of compounds. In the next step of this SAR study we will exchange the methylen/gold center by other metals. Furthermore, investigations are in progress to get deeper insight into the mechanism of action of bisNHC–metal complexes.

Experimental

Chemistry

General

The following instrumentation was used: ^1H or ^{13}C NMR spectra: Bruker ADX 400 spectrometer operated at 400 or 100 MHz (internal standard, tetramethylsilane); Electron impact (EI) MS spectra: Varian CH-7A (70 eV) spectrometer; ESI-TOF spectra: Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA; Elemental C, H, N analysis: PerkinElmer 240 B and C analyzer; Melting point: capillary tube; uncorrected. Chemicals were obtained from Sigma–Aldrich (Germany) and used without further purification. Reactions were all monitored by TLC on silica gel plates 60 F254 (Merck, Darmstadt, Germany), visualized by UV light. Column chromatography was performed with Merck silica gel 60H, grain size <0.063 mm, 230 mesh ASTM (Darmstadt, Germany).

1-Ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazole (**1**) was synthesized according to Refs. [6, 7].

*Bis[1-ethyl-4,5-bis(4-methoxyphenyl)-1H-imidazol-2-yl]-methanone **2***

A solution of 1-ethyl-4,5-bis(4-methoxyphenyl)-1*H*-imidazole (**1**) (20.0 g, 65 mmol) in dry THF (200 mL) was cooled to −45°C and *n*-BuLi (2.5 M in hexane) (26 mL, 65 mmol) was slowly added. The yellow solution was stirred at −45°C for 30 min and then cooled to −80°C. Diethylcarbonate (3.8 g, 32.5 mmol) was added dropwise. The color of the solution changed from pale yellow to purple. After the reaction mixture was allowed to warm to −20°C during 5 h, the reaction was quenched by addition of H_2O (10 mL). After warming to rt, THF was removed *in vacuo* and the residue extracted with CH_2Cl_2 (3 × 50 mL). The CH_2Cl_2 layer was extracted, dried (Na_2SO_4), filtered, and evaporated to give a brown orange oil. The pure product was obtained as colorless crystals after recrystallization from acetone, and from the resulting mother liquor after slow evaporation in air. Yield: 11.2 g (54.0%) of a white solid (mp 203–205°C); MS *m/z*: 642 $[M]^+$; ^1H NMR ($CDCl_3$): δ 1.35 (t, 6H, CH_2CH_3, J = 7.2 Hz), 3.77 (s, 6H, OCH_3), 3.89 (s, 6H, OCH_3), 4.23 (q, 4H, CH_2CH_3, J = 7.2 Hz), 6.77 (d, 4H, ArH, J = 8.8 Hz), 7.02 (d, 4H, ArH, J = 8.8 Hz), 7.32 (d, 4H, ArH, J = 8.8 Hz), 7.49 (d, 4H, ArH, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$): δ 16.8 (s, CH_3); 40.9 (s, CH_2); 55.1, 55.2 (s, OCH_3); 113.5, 114.5, 122.8, 126.9, 128.3, 128.6, 132.1 (s, C_{Ar}); 140.0, 142.4 (s, NCN); 158.5, 160.2 (s, C_{Ar}); 174.6 (s, CO). Anal. calcd for $C_{39}H_{38}N_4O_5$: C, 72.88; H, 5.96; N, 8.72. Found C, 72.64; H, 5.71; N, 8.81.

*Bis[1-ethyl-4,5-bis(4-methoxyphenyl)-1H-imidazol-2-yl]-methane **3***

Compound **2** (6.75 g, 10.5 mmol) and KOH (2 g, 35.6 mmol) were placed into a Schlenk flask. Hydrazine hydrate (17.5 mL, 360 mmol) was added and the flask immersed into an oil bath. On heating a yellow solid began to form at 60°C. The reaction mixture became clear and almost colorless between 110 and 120°C. Stirring at 120°C was continued for 1 h. The temperature was then raised to 150°C and stirring was continued for 3 h at this temperature. Some white solid precipitated at the end of this period. The reaction mixture was allowed to cool to rt during which a white waxy solid formed. At this point all following operations were carried out in air. CH_2Cl_2 (40 mL) was added and the solution transferred to a separatory funnel. The CH_2Cl_2 layer was separated and the remaining light brown liquid extracted with CH_2Cl_2 (2 × 40 mL). The pooled CH_2Cl_2 layers were washed twice with H_2O (2 × 15 mL) to remove excess hydrazine hydrate. The combined H_2O extracts were then repeatedly extracted with CH_2Cl_2 (6 × 20 mL). The combined CH_2Cl_2 solutions were dried (Na_2SO_4), filtered, and rotary evaporated to give an off-white solid. The product was recrystallized from THF, and colorless rhombic crystals separated on cooling to rt. Yield: 4.84 g (73.3%) of a white solid (mp 130–131°C); MS *m/z*: 628 $[M]^+$; ^1H NMR ($CDCl_3$): δ 1.26 (t, 6H, CH_2CH_3, J = 7.2 Hz), 3.76 (s, 6H, OCH_3), 3.80 (q, 4H, CH_2CH_3, J = 7.2 Hz), 3.87 (s, 6H, OCH_3), 6.75 (d, 4H, ArH, J = 8.8 Hz), 6.78 (d, 4H, ArH, J = 8.8 Hz), 7.24 (d, 4H, ArH, J = 8.8 Hz), 7.40 (d, 4H, ArH, J = 8.8 Hz), 7.58 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$): δ 16.3 (s, CH_3); 40.0 (s, CH_2); 49.9 (s, CH_2); 55.0, 55.2 (s, OCH_3); 113.5, 114.5, 122.8, 127.1, 127.5, 127.7, 132.0, (s, C_{Ar}); 135.6, 137.7 (s, NCN); 156.1, 159.7 (s, C_{Ar}). Anal. calcd for $C_{39}H_{40}N_4O_4$: C, 74.50; H, 6.41; N, 8.91. Found C, 74.55; H, 6.52; N, 8.73.

*Bis[1,3-diethyl-4,5-bis(4-methoxyphenyl)imidazol-2-ylidene]methane dibromide **4***

Ethyl bromide (2.7 g, 25.0 mmol) and **3** (3.14 g, 5.0 mmol) were combined and refluxed in 50 mL of acetonitrile for 48–72 h. Removal of the solvent under reduced pressure gave the crude product which was washed with Et_2O (3 × 30 mL) and dried *in vacuo*. Yield: 3.29 g (77.6%) of a white solid (mp 149–151°C); ESI-MS *m/z*: 686 $[M-2Br]^{2+}$; ^1H NMR (DMSO-d_6): δ 1.30 (t, 12H, CH_2CH_3, J = 7.2 Hz), 3.77 (s, 12H, OCH_3), 4.07 (q, 8H, CH_2CH_3, J = 7.2 Hz), 7.01 (d, 8H, ArH, J = 8.4 Hz), 7.33 (d, 8H, ArH, J = 8.8 Hz), 9.40 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$): δ 15.9 (s, CH_3); 43.2 (s, CH_2); 55.4 (s, OCH_3); 114.8, 116.9, 131.5, 131.8 (s, C_{Ar}); 136.2 (s, NCHN); 160.9 (s, C_{Ar}). Anal. calcd for $C_{43}H_{50}Br_2N_4O_4$: C, 61.00; H, 5.95; N, 6.62. Found C, 61.00; H, 5.82; N, 6.77.

*Bis[1-ethyl-4,5-bis(4-hydroxyphenyl)-1H-imidazol-2-yl]-methane **5***

A solution of **3** (1.00 mmol, 0.63 g) in 20 mL of dry CH_2Cl_2 was cooled to −60°C. At this temperature BBr_3 (4.5 mmol, 1.12 g) in 5 mL of dry CH_2Cl_2 was added under N_2 atmosphere. Then the reaction mixture was allowed to warm to rt and was stirred for further 48 h. After cooling the reaction mixture with an ice bath, the surplus of BBr_3 was hydrolyzed three times with methanol and the phenolic product was dissolved in 0.1 N NaOH. The alkaline water phase was filtered and the pH was adjusted to 8 with 2 N HCl. The precipitate was collected by suction filtration and washed with CH_2Cl_2 and acetone to give the product. Yield: 0.374 g (65.5%) of a red solid (mp 304–306°C); MS *m/z*: 572 $[M]^+$; ^1H NMR (DMSO-d_6): δ 1.24 (t, 6H, CH_2CH_3, J = 7.2 Hz), 4.19 (q, 4H, CH_2CH_3, J = 7.2 Hz), 6.69 (d, 4H, ArH, J = 8.4 Hz), 6.96 (d, 4H, ArH, J = 8.4 Hz), 7.25 (d, 4H, ArH, J = 8.4 Hz), 7.30 (d, 4H, ArH, J = 8.4 Hz), 9.62 (s, 2H, CH_2), 10.03 (s, 4H, OH, exchangeable

by D_2O). Anal. calcd for $C_{35}H_{32}N_4O_4$: C, 73.41; H, 5.63; N, 9.78. Found C, 73.54; H, 5.32; N, 9.55.

Bis[1,3-diethyl-4,5-bis(4-hydroxyphenyl)-imidazol-2-ylidene]methane dibromide **6**

Ethyl bromide (0.27 g, 2.5 mmol) and **5** (0.286 g, 0.50 mmol) were combined and refluxed in 20 mL of acetonitrile for 48–72 h. Removal of the solvent under reduced pressure gave the crude product which was washed with Et_2O (3 × 10 mL) and dried *in vacuo*. Yield: 0.269 g (68.2%) of a red solid (mp 295–297°C); ESI-MS *m/z*: 630 $[M-2Br]^{2+}$; ^1H NMR (DMSO-d_6): δ 1.21 (t, 12H, CH_2CH_3, $J = 7.2$ Hz), 4.15 (q, 8H, CH_2CH_3, $J = 7.2$ Hz), 6.84 (d, 4H, ArH, $J = 8.0$ Hz), 6.95 (d, 4H, ArH, $J = 8.0$ Hz), 7.22 (d, 4H, ArH, $J = 8.4$ Hz), 7.27 (d, 4H, ArH, $J = 8.4$ Hz), 9.46 (s, 2H, CH_2), 9.93 (s, 4H, OH, exchangeable by D_2O). Anal. calcd for $C_{39}H_{42}Br_2N_4O_4$: C, 59.25; H, 5.35; N, 7.09. Found C, 59.15; H, 5.53; N, 6.94.

Biological activity

Cell culture

The human MCF-7 and MDA-MB 231 breast cancer and HT-29 colon cancer cell lines were obtained from the American Type Culture Collection. All cell lines were maintained as a monolayer culture in L-glutamine containing Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (PAA Laboratories, Austria), supplemented with 5% fetal bovine serum (FBS; Biochrom, Germany) in a humidified atmosphere (5% CO_2) at 37°C.

Cytotoxicity

The experiments were performed according to established procedures with some modifications [15, 16]. In 96-well plates, 100 μL of a cell suspension in culture medium at 7500 cells/mL (MCF-7 and MDA-MB 231) or 3000 cells/mL (HT-29) were plated into each well and were incubated for 3 days under culture conditions. After addition of various concentrations of the test compounds, cells were incubated for up to appropriate incubation time. Then the medium was removed, the cells were fixed with glutardialdehyde solution 1% and stored under phosphate buffered saline (PBS) at 4°C. Cell biomass was determined by a crystal violet staining, followed by extracting of the bound dye with ethanol, and a photometric measurement at 590 nm. Mean values were calculated and the effects of the compounds were expressed as % Treated/$Control_{corr}$ values according to the following equations:

$$\frac{T}{C_{corr}}[\%] = \frac{T - C_0}{C - C_0} \times 100$$

(C_0 control cells at the time of compound addition; *C* control cells at the time of test end; *T* probes/samples at the time of test end).

The IC_{50} value was determined as the concentration causing 50% inhibition of cell proliferation and calculated as mean of at least two or three independent experiments (OriginPro 8).

Antimicrobial test

The experiments were performed according to agar diffusion test with some modifications [6, 19]. Indicator bacteria were grown in LB at 30°C (*E. amylovora* Ea 273) or 37°C (*E. coli* DH5α, *B. subtilis* 168, and *B. megaterium*). When cell concentration of bacteria was up to 4×10^7 CFU/mL, 0.5 mL of bacteria suspensions were mixed with 20 mL of melting LB agar and cooled below 60°C to prepare the plates. Fifty microliters of a sterile solution (H_2O/DMSO 999:1 v/v) in various concentrations was loaded into the wells (diameter: 5 mm) and punched in indicator bacteria plates which were then incubated at 30°C overnight to observe the growth inhibition effects. Equal amount of sterilized water (containing 0.1% DMSO) was also loaded into indicator bacteria plates as a negative control. The inhibition zone diameter was then measured and recorded.

This work was supported by the China Scholarship Council (File No. 2008613021) and the Deutsche Forschungsemeinschaft (DFG) within FOR 630 "Biological function of organometallic compounds".

The authors have declared no conflict of interest.

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