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Novel gallium(III) complexes containing phthaloyl derivatives of neutral aminoacids with apoptotic activity in cancer cells

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Dedicated to Prof. G. Jaouen on occasion of his 65th birthday.

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ABSTRACT

The reaction of *N*-phthaloylglycine and *N*-phthaloyl-DL-alanine with trimethylgallium (1:1) yielded the dinuclear complexes $[Me_2Ga(\mu\text{-}O_2CCH_2N(CO)_2C_6H_4)]_2$ (**1**) and *RS*-$[Me_2Ga(\mu\text{-}O_2CCHMeN(CO)_2C_6H_4)]_2$ (**2**), respectively. The molecular structure of **2** was determined by X-ray diffraction studies. The cytotoxic activity of the organogallium(III) complexes (**1** and **2**) was tested against human tumour cell lines 8505C anaplastic thyroid cancer, A253 head and neck tumour, A549 lung carcinoma, A2780 ovarian cancer, DLD-1 colon carcinoma and compared with that of cisplatin.

The best response of the synthesized gallium complexes, compared with that of cisplatin, was observed against 8505C anaplastic thyroid cancer and DLD-1 colon carcinoma, while the best IC_{50} values were found for A253 head and neck carcinoma. While the studied carboxylic acids show no proliferative activity, complexes **1** and **2** present very similar cytotoxic activity against all the studied cancer cell lines (IC_{50} from ca. 5 to 25 μM). The cytotoxicities of complexes **1** and **2** are in all cases higher than that presented by gallium(III) nitrate. In addition DNA laddering method showed that treatment of the studied cell lines with IC_{90} doses of **1** and **2** resulted in the induction of apoptotic mode of cell death.

1. Introduction

Platinum-based chemotherapy has been intensively studied over the last 30 years [1–3], however, and due to the impact of the high number of side effects in cancer patients, the interests in this field have shifted to non-platinum metal-based agents [4–10]. In this context, a wide variety of complexes based on different main group and transition metals are being intensively studied [4–10].

One of the studied metals is gallium, due to the analogy of Ga(III) ion with Fe(III) in electronegativity, electron affinity, ionic radius and coordination geometry [11]. In addition, Ga(III) and Fe(III) present quite similar Lewis bases affinity [12], which suggest that Ga(III) ion follows biochemical routes similar to those found in iron metabolism. However, gallium in oxidation state 2+ is energetically unfavourable and, in physiological conditions, oxidation state 1+ is for gallium almost impossible. Redox chemistry is, therefore, not possible for Ga(III) in biological mediums. Thus, gallium presents antitumour effects in the form of simple salts such as gallium nitrate, and interferes with cellular iron metabolism [11,13–16].

The mechanism of action of gallium complexes in anticancer tests, have been briefly studied, and have concluded that gallium affects cellular acquisition of iron by a competitive binding to transferrin, leading to a large amount of cellular gallium uptake [17]. In addition, it presents inhibitory effects on acidification of endosomes [18]. Other studies have focused on the interactions with transferrin receptor and the mechanism of permeation into the cell [19,20].

Once in the cell, gallium competes with Fe^{3+} to bind to ribonucleotide reductase (a key enzyme for DNA synthesis) and this has been considered the most likely route in anticancer action [21]. Some other insights into the mechanism and the application of gallium nitrate as anticancer drug have been reported [22–27].

However, gallium nitrate hydrolyzes very easily in biological mediums towards formation of non-soluble gallium oxides blocking the absorption and membrane permeation of gallium ion. Therefore, many studies on the application of gallium complexes bearing N-, O- or S-donor ligands in anticancer tests have been

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carried out [28–38], in order to find suitable ligands that stabilize gallium against hydrolysis increasing the cytotoxicity in cancer cells. From all these complexes, carboxylate gallium(III) compounds are missing, and only structural properties regarding these compounds have been reported [39–44].

As many of our recent research is centred on the synthesis, characterization and cytotoxic properties of metal-based anticancer drugs [45–49], and in view of the lack of studies about carboxylate gallium(III) complexes in the context of medicinal chemistry [50], as well as the limited investigations concerning interactions of gallium complexes with aminoacid derivatives [51,52], here, we present the synthesis, characterization, antitumoural activity and evaluation of the mode of cell death induced by novel gallium(III) complexes containing *N*-phthaloyl derivatives of neutral aminoacids such as glycine and DL-alanine.

2. Results and discussion

2.1. Synthesis and spectroscopical studies

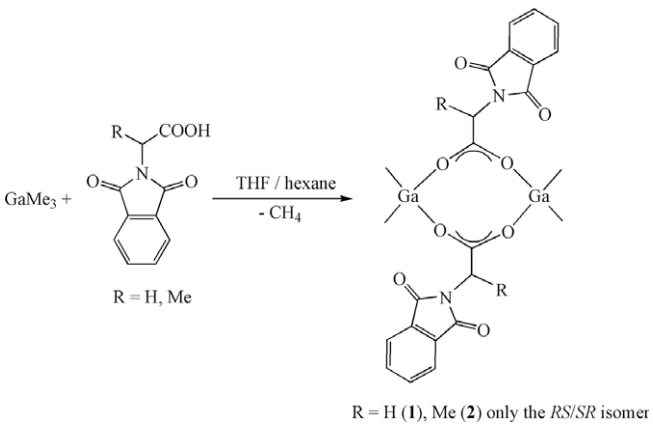
The organogallium compounds $[Me_2Ga(\mu\text{-}O_2CCH_2N(CO)_2C_6H_4)]_2$ (**1**) and *RS*-$[Me_2Ga(\mu\text{-}O_2CCHMeN(CO)_2C_6H_4)]_2$ (**2**) have been prepared by the reaction of *N*-phthaloylglycine and *N*-phthaloyl-DL-alanine with trimethylgallium (Scheme 1).

The elemental analysis, NMR, mass and IR spectra showed that **1** and **2**, isolated as crystalline solids, were of high purity. In complex **2**, the existence of two stereocentres in the molecules should lead to formation of the two diastereoisomers *RS*/*SR* and *RR*/*SS*, however, NMR spectroscopy confirmed the presence of only one of the two possible diastereoisomers in the final product, because only one set of signals was observed. Confirmation of the isolation of the *RS*/*SR* diastereoisomer was also confirmed by X-ray diffraction studies.

In the ^{1}H NMR spectra of **1** and **2** a singlet at ca. −0.3 ppm, corresponding to the protons of the methyl groups attached to gallium, was observed. In addition to this signal, two multiplets at ca. 7.7 and 7.9 ppm assigned to the aromatic protons of the *N*-phthaloyl group were observed.

For **1**, one singlet at 4.44 ppm corresponding to the $-CH_2-$ protons was observed, while in the case of **2**, a doublet at 1.65 and a quadruplet at 4.94 were assigned for the $CH-CH_3$ moiety of the alanine fragment.

$^{13}C\{^1H\}$ NMR spectra for **1** and **2** showed the expected signal for the carbon atoms of the methyl groups attached to gallium at ca. −6 ppm. The signals corresponding to the carbons of the COO moiety of the carboxylate ligands were observed at ca. 171 ppm only with long accumulation and relaxation times. This is maybe due to the very high quadrupolar moment of the gallium nucleus.



Scheme 1.

The $^{13}C\{^1H\}$ NMR spectrum of **1** and **2** showed also three signals between 123 and 134 ppm assigned to the carbon atoms of the phenyl groups as well as one signal at ca. 167 ppm corresponding to the carbon atoms of the carbonyl group. For the carbon atom of the methylene group of the glycine moiety, the $^{13}C\{^1H\}$ NMR spectrum of **1** show a signal at 41.9 ppm while for **2**, the alanine fragment exhibits two signals at ca. 14.1 and 49.4 ppm.

In the infra-red spectra both free ligands show a strong band at ca. 1675 cm^{-1} corresponding to the vibration frequency υ(C=O) of COOH indicating the existence of intermolecular C=O···H–O hydrogen bonds [53]. After deprotonation and binding to gallium atoms, these bands are replaced by strong bands in two different regions at ca. 1580 and 1400 cm^{-1}. These bands correspond to the asymmetric and symmetric vibrations of the COO moiety, respectively. The difference between the asymmetric and symmetric vibrations of less than 200 cm^{-1}, indicate bidentate coordination of the carboxylate ligand [54]. The IR spectrum of **1** and **2** shows also medium absorptions assigned to the Ga-O and Ga-C vibrations at ca. 605 and 530 cm^{-1}.

Both organogallium complexes were also characterized by ESI-MS. The mass spectra did not show the molecular ion peaks, instead, fragments with two gallium atoms and one ligand were observed. For complexes **1** and **2** peaks corresponding to trinuclear species such as $[Ga_3Me_6(O_2CCHRN(CO)_2C_6H_4)_2]^+$ (R = H or Me), were also observed. Low temperature ^{1}H NMR spectra of **2** were recorded, however, no relevant information from these experiments was obtained.

The stability in air of all the complexes in DMSO solutions was also studied by NMR spectroscopy. After 2 days in d_6-DMSO solutions with up to 20% water, no evidence of decomposition or evolution to other products was observed. Attempts to prepare DMSO/water solutions containing more than 30% water, led to the precipitation of the complex.

These studies, suggest a relatively high stability of **1** and **2** in solution, making these complexes very good candidates for anticancer tests.

2.2. Structural studies

The molecular structure of compound **2** consists of a centrosymmetric dimer of two $GaMe_2$ units bridged by two carboxylate groups. This is consistent with previous reports of alkylgallium carboxylate complexes. The crystal structure of **2** (Fig. 1) shows only one of the possible two isomers *RS*. **2** crystallizes in the triclinic space group $P\bar{1}$ with half of the molecule in the asymmetric unit and the other half being generated by symmetry operations. The molecular structure of **2** reveals that the gallium atoms are tetracoordinated in a distorted tetrahedral geometry; with two methyl groups and the two different oxygen atoms of two different carboxylate ligands bound to the metal. The angles C(1)–Ga(1)–C(2) (136.4(1)°) and O(1)–Ga(1)–O(2) (98.61(6)°) are quite distorted from the ideal tetrahedral geometry. The carboxylate ligand is symmetrically bidentate to two different gallium units with bond lengths Ga(1)–O(1) and Ga(1A)–O(2) of 1.961(1) and 1.963(2) Å, which are in the range for other reported for similar complexes [41–44]. The C(3)–O(1)–Ga(1) and C(3)–O(2A)–Ga(1A) angles of 134.1(2) and 125.8(2)° are also consistent with the symmetrical coordination of the carboxylate ligand. The very similar bond lengths for C(3)–O(1) and C(3)–O(2A) of 1.251(1) Å both, show multiple bond character and symmetric coordination of the carboxylate ligand.

The deviation of Ga(1) and Ga(1A) from the meanplane formed by O(1)–O(2A)–C(3)–C(4) is ca. 1.35 and 0.38 Å, respectively. The distance between the two metal centres [M(1)···M(1A)] of ca. 4.35 Å is comparable to the range observed for related alkylgallium (ca. 4.35–4.68 Å) carboxylate complexes [41–44].

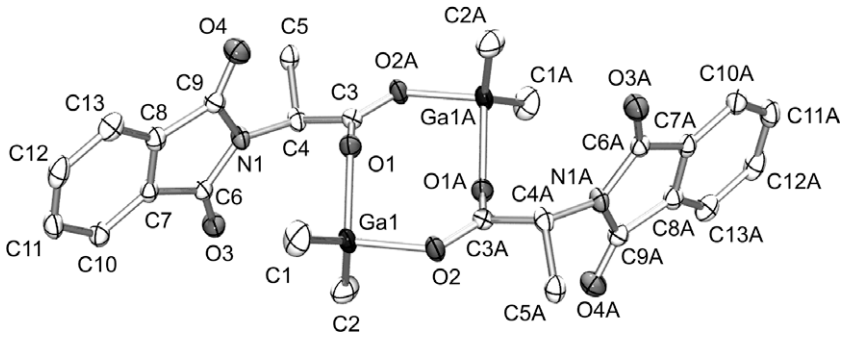


Fig. 1. Molecular structure and atom-labelling scheme for **2** with thermal ellipsoids at 50% probability (hydrogen atoms are omitted for clarity).

Table 1
Selected bond lengths (Å) and angles (°) for **2**.

	2
Ga(1)–O(1)	1.9606(14)
Ga(1)–O(2)	1.9632(15)
Ga(1)–C(1)	1.933(2)
Ga(1)–C(2)	1.934(2)
C(3)–O(1)	1.255(2)
C(3)–O(2A)	1.255(2)
C(1)–Ga(1)–C(2)	136.36(12)
C(1)–Ga(1)–O(1)	103.57(9)
C(2)–Ga(1)–O(1)	104.51(9)
C(1)–Ga(1)–O(2)	100.61(10)
O(1)–Ga(1)–O(2)	98.61(6)
O(1)–C(3)–O(2A)	123.8(2)
C(3)–O(1)–Ga(1)	134.12(14)
C(3)–O(2A)–Ga(1A)	125.84(14)

Symmetry transformations used to generate equivalent atoms: $-x, -y, -z+2$.

Both gallium atoms, the four oxygen atoms and two carbon atoms of the two carboxylate fragments, form a puckered eight-membered ring. The puckering of the $M_2O_4C_2$ ring is a result of folding of the eight-membered ring along the two inter-ligand O···O vectors. The extent of folding (θ_{ring}), previously defined as the angle between the GaO_2 planes and the O_4C_2 plane [41,55], is 43.5° as in the case of most of the related complexes [41,55]. Selected bond lengths and angles for **2** are summarized in Table 1.

2.3. Cytotoxic studies

The *in vitro* cytotoxicities of carboxylic acids, **1** and **2** as well as gallium(III) nitrate against human tumour cell lines 8505C anaplastic thyroid cancer, A253 head and neck carcinoma, A549 lung carcinoma, A2780 ovarian cancer, DLD-1 colon carcinoma were determined by using the sulforhodamine-B microculture colorimetric assay [56]. The IC_{50} values of the carboxylic acids, complexes **1** and **2**, gallium(III) nitrate and cisplatin are summarized in Table 2.

The present study reveals that while carboxylic acids presented no antiproliferative effect in the studied concentrations, cytotoxicities of gallium(III) complexes containing *N*-phthaloyl derivatives of aminoacids (**1** and **2**) are, in all cases, higher than those observed for gallium(III) nitrate. Complexes **1** and **2** showed a dose-dependent antiproliferative effect toward all cancer cell lines (Fig. 2). Gallium compounds present higher IC_{50} values than those of cisplatin, however, the obtained values are comparable to those reported for other gallium complexes [36].

The best response (compared with that of cisplatin) of the synthesized gallium complexes, was observed against 8505C anaplastic thyroid cancer and DLD-1 colon carcinoma, with ratios of 0.35 and 0.32 cells gallium complex/cisplatin, respectively. Complexes **1** and **2** present very similar activity against all the studied cancer cell lines. **1** (14.12 ± 3.74 μM) is slightly better than **2** (18.24 ± 4.28 μM) against 8505C anaplastic thyroid cancer, A253 head and neck tumour (**1**: 5.72 ± 0.29 μM; **2**: 6.59 ± 0.34 μM) A2780 ovarian cancer (**1**: 13.97 ± 0.74 μM; **2**: 15.88 ± 0.36 μM) and DLD-1 colon carcinoma (**1**: 15.68 ± 0.11 μM; **2**: 17.94 ± 0.23 μM), while against A549 lung carcinoma (**1**: 25.58 ± 4.73 μM; **2**: 26.31 ± 8.31 μM) both complexes can be considered identical.

The studied complexes have a very high structural analogy, thus, one cannot expect a notable difference in the cytotoxic activity.

2.4. Apoptosis studies

Apoptosis and cell mediated cytotoxicity are characterized by a fragmentation of the genomic DNA. These DNA fragments have a length of about 180 bp or multiples of this number (360, 540, 720,...), this is, the characteristic DNA-length of a nucleosome (DNA–histone complex). Endonucleases cleave selectively DNA at sites located between nucleosomal units (linker DNA). In agarose gel electrophoresis of these DNA fragments are resolved to a distinctive ladder pattern. To test whether complexes **1** and **2** induced cell death mediated by apoptosis, floating cells from 8505C, A253, A549, A2780 and DLD-1 after 24 h treatment with the IC_{90} concentrations were collected and analyzed by DNA laddering technique.

Table 2
IC_{50} (μM) for the 96 h of action of the studied compounds, gallium(III) nitrate and cisplatin on 8505C anaplastic thyroid cancer, A253 head and neck tumour, A549 lung carcinoma, A2780 ovarian cancer, DLD-1 colon carcinoma determined by sulforhodamine-B microculture colorimetric assay.

Compound	IC_{50} ± SD				
	8505C	A253	A549	A2780	DLD-1
1	14.12 ± 3.74	5.72 ± 0.29	26.31 ± 8.31	13.97 ± 0.74	15.68 ± 0.11
2	18.24 ± 4.28	6.59 ± 0.34	25.58 ± 4.73	15.88 ± 0.36	17.94 ± 0.23
N-phthaloylglycine	>100	>100	>100	>100	>100
N-phthaloyl-DL-alanine	>100	>100	>100	>100	>100
$Ga(NO_3)_3$	95.45 ± 10.07	33.91 ± 0.31	>100	32.00 ± 1.15	>100
Cisplatin	5	0.8	1.5	0.5	5

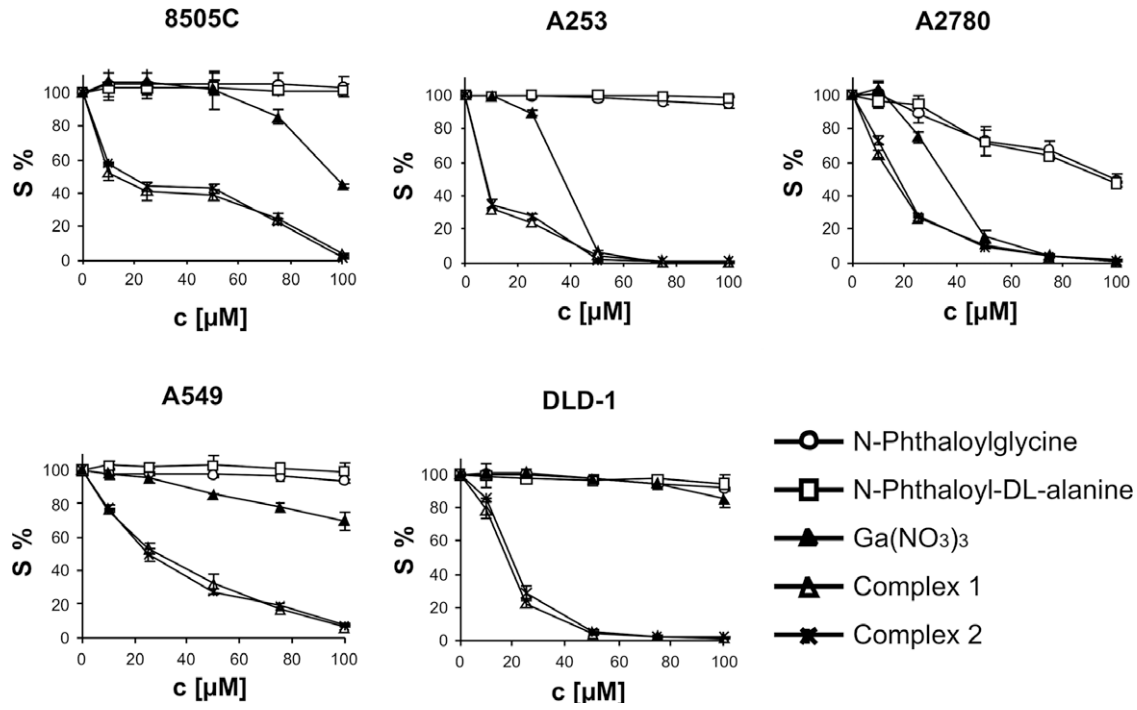


Fig. 2. Representative graphs show survival of 8505C, A253, A549, A2780 and DLD-1 cells grown for 96 h in the presence of increasing concentrations of the studied carboxylic acids, gallium(III) nitrate and complexes **1** and **2**.

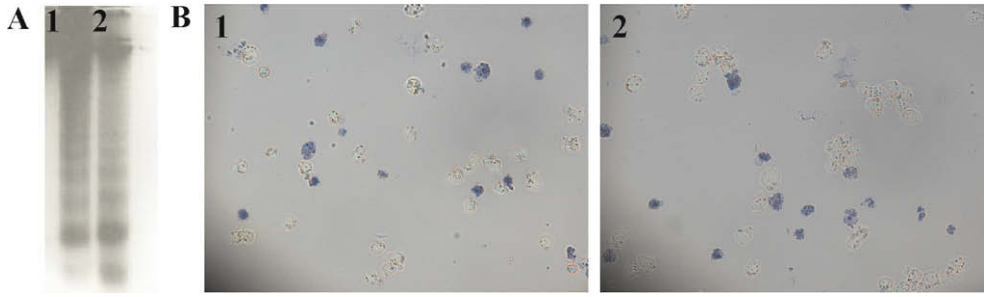


Fig. 3. Confirmation of apoptotic cell death with DNA laddering as an example in DLD-1 (A) and with trypan blue in A2780 cells (B) induced by compounds **1** and **2**.

In all five investigated cell lines with both compounds, **1** and **2**, typical DNA laddering was observed (Fig. 3A). Furthermore, 8505C, A253, A549, A2780 and DLD-1 cells were exposed to IC_{90} doses, the ability to exclude trypan blue and detached cells were investigated for. The treatment with IC_{90} doses of **1** and **2** resulted in the induction of apoptotic cell death, in which floating cells showed the ability to exclude the blue dye indicating that **1** and **2** caused cell death by the induction of apoptosis (Fig. 3B). The blue stained cells have undergone secondary necrosis.

3. Experimental

3.1. General manipulations

All experiments were performed under an atmosphere of dry nitrogen using standard Schlenk techniques. Solvents were distilled from the appropriate drying agents and degassed before use. NMR spectra were recorded on a Bruker AVANCE DRX 400 spectrometer. ^{1}H NMR (400.13 MHz): internal standard solvent, external standard TMS; $^{13}C\{^1H\}$ NMR (100.6 MHz): internal standard solvent, external standard TMS. IR spectra: KBr pellets were prepared in a nitrogen-filled glove box and the spectra were recorded on a Perkin–Elmer System 2000 FTIR spectrometer in the range 350–4000 cm^{-1}. ESI-MS were recorded with a FT-ICR MS Bruker-Daltonics (APEX II, 7 Tesla, MASPEC II), and solutions of ca. 1 mg/mL of the compounds in a mixture of dry CH_2Cl_2:CH_3CN (1:1) were injected.

Trimethylgallium was obtained from commercial suppliers. All the commercial reagents were used directly, without further purification. *N*-phthaloylglycine and *N*-phthaloyl-DL-alanine were prepared with slight modification of the literature procedure [57].

3.2. Synthesis of $[Me_2Ga(\mu\text{-}O_2CCH_2N(CO)_2C_6H_4)]_2$ (**1**)

2.16 mL (2.59 mmol) of a 1.2 M solution of $GaMe_3$ in hexane were added dropwise at 0 °C during 15 min to a solution of *N*-phthaloylglycine (0.53 g, 2.59 mmol) in THF (30 mL). The reaction mixture was stirred for 30 min at this temperature. The reaction was then stirred at room temperature one more hour. The solvent was then removed in vacuo and the resulting solid dissolved in toluene (10 mL). The clear solution was concentrated (5 mL) and cooled to −30 °C to give a microcrystalline solid of the title complex which was isolated by filtration and washed twice with hexane (5 mL). Yield: 0.65 g, 83%. FT-IR (KBr): 1564 (s) (υ_a COO^-),

1413 (s) (υ_s COO^-), 611 (m) (υ Ga–O), 531 (m) (υ Ga–C); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ −0.31 (s, 12H, Ga*Me*$_2$), 4.44 (br s, 4H, C*H*$_2$ of Gly), 7.86 (br m, 4H, aromatic H in Ph), 8.05 (br m, 2H, aromatic H in Ph); ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$, 25 °C): δ −7.9 (Ga*Me*$_2$), 41.9 (*C*H$_2$ of Gly), 123.3, 133.8 (*C*H of Ph), 132.1 (*ipso*-C of Ph), 167.4 (*C*ON), 171.0 (*C*OO); ESI-MS: solution in CH_3CN:CH_2Cl_2 (1:1); *m*/*z*: 706.97 [$Ga_3Me_6(O_2CCH_2N(CO)_2C_6H_4)_2$], 403.97 [$M^+$−*N*-phthaloylglycine]; Anal. Calc. for $C_{24}H_{24}Ga_2N_2O_8$ (607.9): C, 47.42; H, 3.98. Found: C, 47.22; H, 4.19%.

3.3. Synthesis of RS-[$Me_2Ga(\mu$-$O_2CCHMeN(CO)_2C_6H_4)]_2$ (**2**)

The synthesis of **2** was carried out in an identical manner to **1**. *N*-phthaloyl-DL-alanine (0.57 g, 2.59 mmol) and $GaMe_3$ (1.2 M) (2.16 mL, 2.59 mmol). Yield: 0.75 g, 91%. FT-IR (KBr): 1599 (s) (υ_a COO^-), 1399 (s) (υ_s COO^-), 601 (m) (υ Ga–O), 531 (m) (υ Ga–C); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ −0.31 (s, 12H, Ga*Me*$_2$), 1.65 (d, 6H, $^3J(^1H–^1H) = 6.8$ Hz, C*H*$_3$ of Ala), 4.94 (q, 2H, $^3J(^1H–^1H) = 6.8$ Hz, C*H* of Ala), 7.72 (m, 4H, aromatic H in Ph), 7.85 (m, 4H, aromatic H in Ph); ^{13}C{^{1}H} NMR (100.6 MHz, $CDCl_3$, 25 °C): δ −8.1 (Ga*Me*$_2$), 14.1 (*C*H$_3$ of Ala) 49.4 (*C*H of Ala), 123.4, 133.9 (*C*H of Ph), 131.9 (*ipso*-C of Ph), 167.4 (*C*ON), 170.9 (*C*OO); ESI-MS: solution in CH_3CN:CH_2Cl_2 (1:1); *m*/*z*: 735.01 [$Ga_3Me_6(O_2CCHMeN(CO)_2C_6H_4)_2$], 417.99 [$M^+$−*N*-phthaloyl-DL-alanine]; Anal. Calc. for $C_{26}H_{28}Ga_2N_2O_8$ (635.9): C, 49.10; H, 4.44. Found: C, 48.99; H, 4.59%.

3.4. Data collection and structural refinement of 2

The data of **2** were collected with a CCD Oxford Xcalibur S (λ (Mo Kα) = 0.71073 Å) using ω and φ scans mode. Semi-empirical from equivalents absorption corrections were carried out with SCALE3 ABSPACK [58]. All the structures were solved by direct methods [59]. Structure refinement was carried out with SHELXL-97 [60]. All non-hydrogen atoms were refined anisotropically, and hydrogen atoms were calculated with the riding model and refined isotropically. Table 3 lists crystallographic details.

Table 3
Crystallographic data for **2**.

	2
Formula	$C_{26}H_{28}Ga_2N_2O_8$
Formula weight	635.94
T (K)	130(2)
Crystal system	Triclinic
Space group	$P\bar{1}$
a (Å)	8.5056(8)
b (Å)	9.2159(7)
c (Å)	10.5478(8)
α (°)	95.847(6)
β (°)	110.501(8)
γ (°)	115.460(8)
V (nm^3)	0.66736(10)
Z	1
D_{calc} (Mg m^{-3})	1.582
μ (mm^{-1})	2.070
F(000)	324
Crystal dimension (mm)	0.2 × 0.2 × 0.15
θ Range (°)	2.56–26.37
hkl Ranges	$-8 \le h \le 10$, $-11 \le k \le 11$, $-12 \le l \le 13$
Data/parameters	2685/175
Goodness-of-fit on F^2	0.998
Final *R* indices [$I > 2\sigma(I)$]	$R_1 = 0.0268$, $wR_2 = 0.0587$
R indices (all data)	$R_1 = 0.0343$, $wR_2 = 0.0598$
Largest difference in peak and hole (e Å^{-3})	0.558 and −0.244

4. In vitro studies

4.1. Preparation of drug solutions

Stock solutions of investigated gallium complexes were made in dimethyl sulfoxide (DMSO, Sigma–Aldrich; carboxylic acids, **1** and **2**) and water (gallium(III) nitrate) at a concentration of 20 mM, filtered through Millipore filter, 0.22 μm, before use, and diluted by nutrient medium to various working concentrations. Nutrient medium was RPMI-1640 (PAA Laboratories) supplemented with 10% fetal bovine serum (Biochrom AG) and penicillin/streptomycin (PAA Laboratories).

4.2. Cell lines and culture conditions

The cell lines 8505C, A253, A549, A2780 and DLD-1 were included in this study. All these cell lines were kindly provided by Dr. Thomas Müller, Department of Hematology/Oncology, Martin Luther University of Halle-Wittenberg, Halle (Saale), Germany. Cultures were maintained as monolayer in RPMI-1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37 °C in a humidified atmosphere of 5% (*v*/*v*) CO_2.

4.3. Cytotoxicity assay

The cytotoxic activities of the gallium compounds were evaluated using the sulforhodamine-B (SRB, Sigma–Aldrich) microculture colorimetric assay [56]. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the studied compounds for 96 h. Final concentrations achieved in treated wells were 10, 25, 50, 75, and 100 μmol/L. Each concentration was tested in three triplicates on each cell line. The final concentration of DMSO solvent never exceeded 0.5%, which was non-toxic to the cells. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After 96 h treatment, the supernatant medium from the 96-well plates was thrown away and the cells were fixed with 10% TCA. For a thorough fixation plates were then allowed to stand at 4 °C. After fixation, the cells are washed in a strip washer. The washing was carried out four times with water using alternate dispensing and aspiration procedures. The plates were then dyed with 100 μL of 0.4% SRB for about 45 min. After dyeing the plates were again washed to remove the dye with 1% acetic acid and allowed to air dry overnight. 100 μL of 10 mM Tris base solutions was added to each well of the plate and absorbance was measured at 570 nm using a 96-well plate reader (Tecan Spectra, Crailsheim, Germany). The IC_{50} values, defined as the concentrations of the compound at which 50% cell inhibition was observed, were estimated from the dose–response curves.

5. Apoptosis tests

5.1. Trypan blue exclusion test

Apoptotic cell death was analyzed by trypan blue dye (Sigma–Aldrich, Germany) on 8505C, A253, A549, A2780 and DLD-1 cell lines. The cell culture flasks with 70–80% confluence were treated with IC_{90} doses of **1** and **2** for 24 h. The supernatant medium with floating cells was collected after treatment, and centrifuged to collect the dead and apoptotic cells. The cell pellet was resuspended in serum free media. Equal amounts of cell suspension and trypan

blue were then mixed and this was observed under a microscope. The cells, which were able to exclude the dye were colourless. Those cells whose cell membrane was destroyed were blue. If the proportion of colourless cells is higher than that of those that are coloured the cell death can be considered as apoptotic.

5.2. DNA fragmentation assay

Determination of apoptotic cell death was performed by DNA gel electrophoresis. 8505C, A253, A549, A2780 and DLD-1 cells were treated with the respective IC_{90} doses of **1** and **2** for 24 h. Floating cells induced by drug exposure were collected, washed with PBS and lysed with lysis buffer (100 mM Tris–HCL pH 8.0; 20 mM EDTA; 0.8% SDS; all from Sigma Aldrich). Subsequently, they were treated with RNAse A at 37 °C for 2 h and proteinase K at 50 °C (both from Roche Diagnostics Chemical Company, Mannheim, Germany). DNA laddering was observed by running the samples on 2% agarose gel followed by ethidium bromide (Sigma–Aldrich) staining.

6. Conclusions

Two novel organogallium(III) complexes containing phthaloyl derivatives of neutral aminoacids have been synthesized and structurally characterized. Metal complexes were tested *in vitro* against human tumour cell lines 8505C anaplastic thyroid cancer, A253 head and neck tumour, A549 lung carcinoma, A2780 ovarian cancer and DLD-1 colon carcinoma. While the carboxylic acids presented no cytotoxicity against the studied cells, complexes **1** and **2** presented antiproliferative effect, which was higher than that presented by gallium(III) nitrate.

In addition, treatment of the 8505C, A253, A549, A2780 and DLD-1 cells with IC_{90} doses of **1** and **2** revealed the mode of cell death by DNA laddering and trypan blue exclusion tests. All the studied cell lines were able to exclude trypan blue and their DNA showed typical fragmentation, indicating that **1** and **2** caused cell death by the induction of apoptosis. Further studies on the apoptosis mechanism of these organometallic complexes will be carried out.

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Appendix A. Supplementary material

CCDC 714454 contains the supplementary crystallographic data for **2**. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2009.02.027.

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