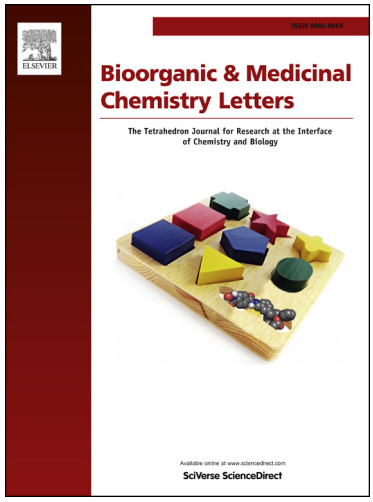

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PII: S0960-894X(13)00580-5
DOI: http://dx.doi.org/10.1016/j.bmcl.2013.04.088
Reference: BMCL 20455

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 18 October 2012
Revised Date: 26 April 2013
Accepted Date: 30 April 2013

Please cite this article as: Zhang, X., Zuo, Z., Tang, J., Wang, K., Wang, C., Chen, W., Li, C., Xu, W., Xiong, X., Yuntai, K., Huang, J., Lan, X., Zhou, H-B., Design, Synthesis and Biological Evaluation of Novel Estrogen-derived Steroid Metal Complexes, *Bioorganic & Medicinal Chemistry Letters* (2013), doi: http://dx.doi.org/10.1016/j.bmcl.2013.04.088

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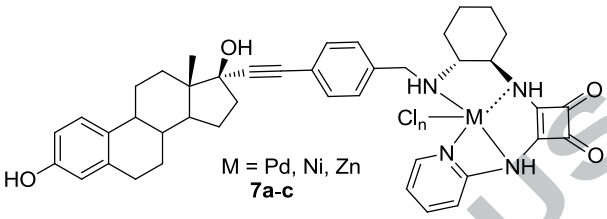

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

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ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
breast cancer
estrogen
squaric acid
metal complex

ABSTRACT

A new series of estrogen-derived metal complexes were synthesized and characterized. The functionalized estrogen receptor ligands were prepared by a four-step synthetic strategy, and then three transition metal Pd, Ni, Zn were introduced readily to give the title metal complexes, in which the squaramide was introduced as ion acceptor for the first time in the development of estrogen-derived metal complexes for estrogen receptor. Upon binding to estrogen receptors, all of the estrogen conjugates exhibited acceptable binding affinity (up to 2.3% relative to Estradiol), and in transcription assays, all the compounds are agonists on ERα. Molecular modeling studies suggest a structural basis for the agonist activity of these compounds.

The steroid hormone estrogens play key roles not only in the maintenance of normal sexual and reproductive function but also in the progression of numerous diseases through regulation of the transcriptional activity of its cognate receptors[1]. Classically, most of the actions of estrogens appear to be exerted via two estrogen receptor (ER) subtypes, denoted ERa and ERβ, which bind as dimers to estrogen-response elements in the regulatory regions of the estrogen-responsive genes and associate with basal transcription factors and coregulators to alter gene expression[2], though recently much evidence has shown some other pathways[3]. Ever since the link between human breast cancer and estrogen emerged over a century ago, people have realized that hormones are key contributors to carcinogenesis in specific cancers[4]. An association between the risk of breast cancer and persistently elevated blood level of estrogen has been consistently found in many studies[5]. Estrogen stimulates the proliferation of breast epithelial cells, and both endogenous and exogenous estrogens have been implicated in the pathogenesis of breast cancer[6].

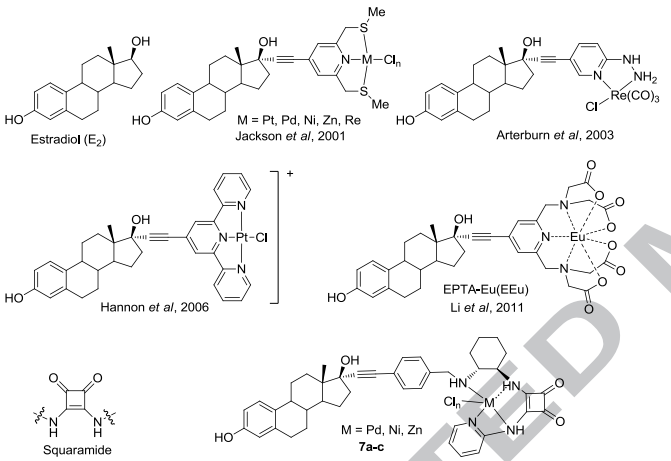
Meanwhile, transition metal centers have been being widely utilized as agents for diagnostic imaging and medical therapeutic treatment both in the clinic and in research programs[7]. In recent decades, a variety of estrogen derivatives labeled with transition metal Pt, Re, Tc, Ru, *etc*[8] have been investigated as targeting agents into ER-positive cells. In addition, metal complex as a radionuclide which has several advantages and played a central role in diagnostic medical imaging for several years. They can be produced using commercially available generator systems combined with other favorable characteristics such as a convenient half-life, which allows for complex synthesis and prolonged imaging. Moreover, the ability of these metal complexes to deliver metal centers across the cellular membrane and into specific cells especially tumor cells is consequently an important goal.

Concerning the metal chelating strategies, a variety of conjugating scaffolds have been reported before[7a-c]. With a good knowledge of the estrogen receptors and theirs ligands, we are very interested in the linkage between the metal ion and the 17α-position of E_2. Previous studies shown that the 17α-position of E_2 can tolerate bulky organometallic species without much loss of the binding affinity to ER[9]. At the same time, an ethynyl linker group between the estrogen 17α-position and the organometallic moiety was proven to maintain an effective ER binding affinity[10]. Estrogenic steroid conjugates containing metal chelates at 17α-position represent an attractive delivery vector for such targeting strategy[11]. Especially, a pyridine ring linked to the ethynyl group at the estrogen 17α-position is often utilized as the key component of the metal chelating groups for either potential imaging agents or SERMs (Selective Estrogen Receptor Modulators, examples shown in **Scheme 1**)[12]. These design ideas are also taken into consideration in our work.

Our recent work, described in this report, focuses on the search for more potent estrogen-derived metal complexes which may have higher binding affinity and desired characteristics including the redox properties, stability and lipophilicity. And also, we are very interested in incorporating the squaramide moiety into the metal complexes. It is known that the squaric acid derivatives have been applied in a wide variety of chemistry fields including bioconjugate chemistry, medicinal chemistry,

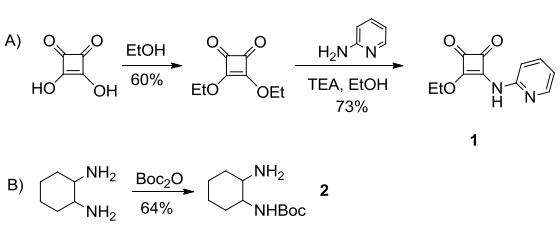
molecular recognition, organometallic chemistry and organocatalysis due to its unique properties [13]. Therefore, we apply the squaramide structure as the core of metal binding unit in our molecular design. On the other hand, since the first application of Oxaliplatin (the first effective platinum-based agent for the treatment of colorectal cancer[13]), great attention has been paid on the complexes containing 1,2-diaminocyclohexane (1,2-DACH) moiety regarding to their anticancer activities[15,16]. It proved to be particularly convenient to use the 1,2-cyclohexane and squaramide as metal chelating moieties. The synthetic accessibility of the title compound is feasible with the advantages of 1,2-diaminocyclohexane and squaramide as building blocks.

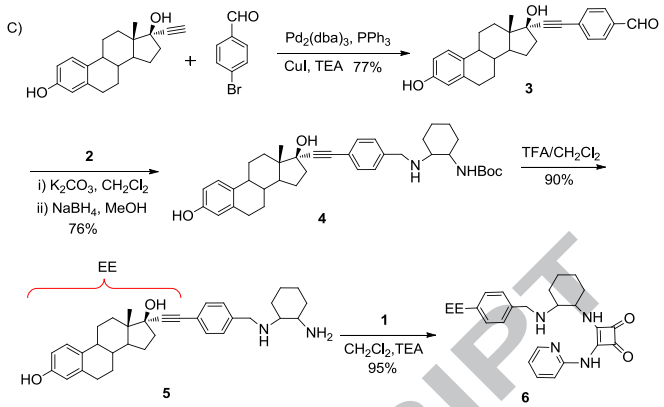
Based on the facts above, we designed and prepared estradiol derived squaramide ligand **6** and its metal complexes **7a-c**. Their binding affinities to the ER-LBD (estrogen receptor-ligand binding domain) were also examined. All of the synthesized compounds exhibited effective binding to the estrogen receptors. The results suggested the promising application of this series of estrogen-derived metal complexes as a novel type of targeting agents for estrogen receptors.



Scheme 1 Representative steroidal-derived metal complexes and title compound **7**

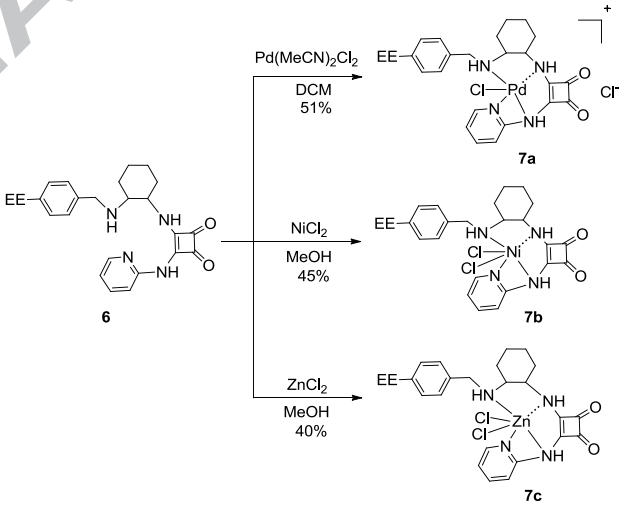
As shown in **Scheme 2**, compounds **1** and **2** were conveniently prepared from squaric acid and 1,2-cyclohexanediamine respectively with moderate yield (**Scheme 2A** and **2B**)[17]. These two compounds comprised the major part of metal chelate moiety. Sonogashira coupling reaction of 17α-ethynylestradiol with *p*-bromobenzaldehyde afforded corresponding product **3** in good yield. Subsequent reductive amination of compound **3** with compound **2** produced compound **4** in 76% yield[18]. Hydrolysis of **4** with 50% TFA/CH_2Cl_2[19] led to compound **5** which then reacted with **2** in EtOH to give rise to the target neutral ligand **6** in 95% yield (**Scheme 2C**).





Scheme 2 Synthesis of the neutral ligand **6**

With the ligand **6** in hand, we tried to prepare a variety of metal complexes **7** (**Scheme 3**)[12a]. For complex **7a**, mixture of ligand **6** and $Pd(MeCN)_2Cl_2$ in anhydrous CH_2Cl_2 solution under argon atmosphere was stirred at ambient temperature overnight. Then the solution was filtered and washed with CH_2Cl_2, which afforded the complex **7a** in 51% yield without further purification. Compounds **7b-c** were synthesized under similar procedures in anhydrous MeOH instead of CH_2Cl_2 with metal source $NiCl_2$ and $ZnCl_2$, respectively. The structures of complexes **7a-c** have been confirmed by IR, NMR and mass spectrometry[20].



Scheme 3 Synthesis of the complexes **7a-c**

Having successfully obtained the complexes **7a-c**, we wondered whether these steroidal compounds would retain binding affinity and transcription activation for the estrogen receptor. The estrogen receptor binding affinities of complexes **7a-c** were measured by a competitive fluorescence polarization binding assay, using both purified human ERα/ERβ-LBD (details shown in ***Supporting Information***)[21]. The binding affinities of the compound **6** and the complexes **7** in this study were summarized in **Table 1**. The lipophilicity value (expressed in log*P*o/w) of the compounds **6** and **7** was also calculated with the Molecular Operating Environment (MOE) package[22]. The log*P*o/w values of complexes **7a-c** were calculated to be 5.38, 6.85 and 6.95, respectively. The compounds **7b** and **7c** were more lipophilic than the free ligand **6** (ca. 6.16), however, **7a** is slightly less hydrophobic than **6** possibly due to its cationic nature. The high lipophilicity of these molecules is anticipated to be a good compromise for a potential pharmaceutical product.

Table 1. Relative Binding Affinity (RBA) of compounds **6** and **7a-c** for ERα and ERβ[a]

Compound[b]	ERα (%)	ERβ (%)
Estradiol	100	100
6	0.65 ± 0.01	4.04 ± 0.38
7a	0.95 ± 0.1	0.85 ± 0.05
7b	2.3 ± 0.5	0.57 ± 0.15
7c	0.88 ± 0.03	< 0.1

[a]Relative binding affinity (RBA) values were determined by competitive binding assay based on fluorescence polarization (Estradiol = 100%). [b]Mean of three experiments ± range.

From the **Table 1** we could see that all synthesized complexes still retained essential binding affinity to both ERα and ERβ (except **7c**). The conjugation of metal ions into the neutral ligand **6** indeed influences their RBA, but the effect can be either positive or negative. Additionally, among the complexes **7a-c**, **7b** (Ni) showed the highest RBA to ERα, but **7a** (Pd) had the best RBA to ERβ. This binding preference may be related to the distinct characteristics of the three transition metal ions.

Compared to the neutral free ligand **6**, the binding affinity of **7a-c** for ERα increased (the highest is 4 fold), while the binding affinity of **7a-c** for ERβ all decreased. Some works reported that the binding affinity of a cationic complex was very likely to drop to some degree when compared to its neutral metal-free ligand[10a]. But Jackson *et al* reported some estrogen derived metal complexes showed higher ER binding affinity than the neutral metal-free ligands[12a].

The transcriptional activity of these compounds was tested by a Luciferase reporter gene assays in human embryonic kidney 293T (HEK-293T) cells (details shown in ***Supporting Information***). The transcriptional activities were summarized in Table 3.

From the **Table 2** we could conclude that in HEK-293T cells, all synthesized compounds were agonists on ERα. Compound **7a** and **7c** showed greater agonist activity than the neutral free ligand **6**. It was clear that this series of compounds could not or just have low efficacy acting as antagonist on ERα.

Table 2. Transcription Activation of compounds **6** and **7a-c** through ERα[a]

Cmpd	Agonist mode		Antagonist mode	
	ERα		ERα	
	EC_{50} (uM)	Eff (%E_2)	IC_{50} (uM)	Eff (%E_2)
6	1	93 ± 6	-	-
7a	0.019	96 ± 11	0.3	31 ± 7
7b	0.00034	38 ± 4	-	-
7c	0.007	95 ± 9	0.0001	28 ± 2

[a] Cells were transfected with full-length human ERα and an estrogen responsive reporter gene. Compounds were tested at 10^{-5} to 10^{-11} M alone (as agonists) or in the presence of 10^{-8} M estradiol (as antagonists). In all cases, 10^{-8} M estradiol alone was set as 100% activation ability.

To further investigate the structure-binding affinity correlations and how those ligands oriented, we performed molecular docking by autodock software. Because of lacking of force field parameters for Ni and Pd, we only modeled the ligands of **6**, **7c** and estradiol into the ligand binding pocket of both activation (PDB code: 2YAT[12d]) and inhibition (PDBcode: 2OUZ[23]) conformations of ERα. Ligand **6** can dock well with both the ERα conformations[24]. The estradiol moiety in compound **6** can superimpose well with estradiol (data not show). As a result, a similar binding model achieved—the A ring forms hydrogen bonding with Glu 353 and Arg 394, the hydroxyl group at 17β position forms a hydrogen bond with His 524 (**Figure 1 A**). The rest part of ligand **6** orients to the beta sheets which opposite to and far from helix 12 (**Figure 1 A**). It seems that ligand **6** has no effect on helix 12. There are four nitrogen atoms near Zn in complex **7c**, after energy minimization in MMFF94 force field, we found that three coordination bonds were formed. Ligand **7c** can dock well with ERα activation conformation (2YAT), but no reasonable orientation was found with ERα inhibitory conformation (2OUZ). The estradiol moiety in compound **7c** binds with ERα activation conformation in the same manner with compound **6** (**Figure 1 B/C**). The binding site of ERα inhibitory conformation getting narrow near the β-sheet and the introduction of Zn making the substitute moiety more rigid, both of the reasons make compound **7c** fail to dock with ERα inhibitory conformation (**Figure 1 D**). Larger molecules tend to receive higher free energy during modeling in autodock, so we adopt energy-mass ratio (energy-mass ratio = 100 * free energy / mass) to represent the binding affinity, and the result show in **Table 3**. Compound **6** got an energy-mass ratio of -2.27 to 2YAT, while -2.31 to 2OUZ and **7c** got -1.81 to 2YAT. It reveals that both compound **6** and **7c** can dock with ERα very well.

Table 3. Energy-mass ratio estradiol and compound **6**[a]

	2YAT	2OUZ
ES	-3.99[a]	-3.48
6	-2.27	-2.31
7c	-1.81	--

[a]energy-mass ratio = 100*free energy/mass.

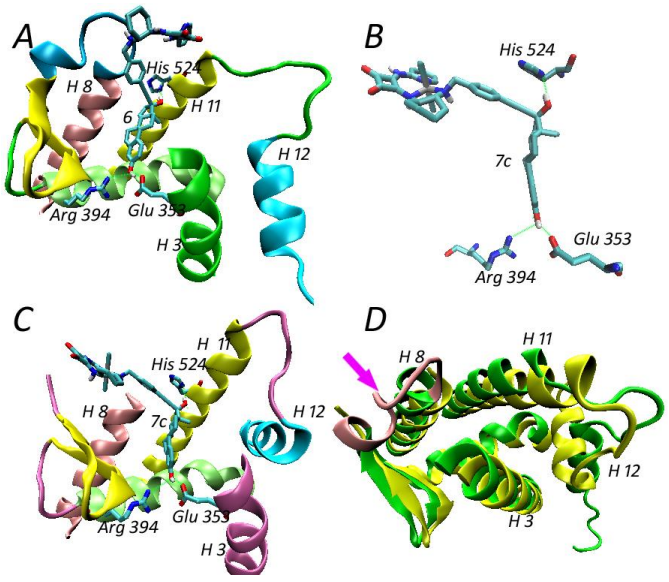


Figure 1. The binding model of compound **6** and **7c** with 2YAT and 2OUZ. A) The binding model of compound **6** with2OUZ; B) His 524, Glu 353 and Arg 394 can form hydrogen bonds with **7c** (the same with compound **6**, data not shown). C) The binding model of compound **7c** with 2YAT. D) Conformation difference

of 2OUZ (green) and 2YAT (yellow), the loop between helix 8 and β-sheet make **7c** failed to dock with 2OUZ.

In this study, we have described the synthesis and biological evaluation of a series of estradiol-derived metal complexes containing an unique squaramide moiety. Three metal ions in the first and second row of transition metal have been incorporated into the neutral ligand. All the designed complexes retain low but effective binding affinity to estrogen receptors, and in transcription assays, all the compounds are agonists on ERα. Molecular modeling studies suggest a structural basis for the agonist activity of these compounds.

Acknowledgments

We are grateful to the NSFC (81172935, 91017005, 30970914), the Program for New Century Excellent Talents in University (NCET-10-0625), Key Project of Ministry of Education (313040), the Scientific and Technological innovative Research Team of Wuhan (2013070204020048), and the Fundamental Research Funds for the Central Universities for support of this research.

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20. **Experimental**: Melting points were obtained on X-4 melting point apparatus (Beijing TECH Instruments, Co., Ltd.) and are uncorrected.IR spectra were measured on a Nicolet 470FT-IR spectrophotometer in the range of 400–4000 cm^{-1}. ^1H NMR and ^{13}C NMR spectra were obtained on Bruker Biospin AV400 (400 MHz) instrument. The chemical shifts are reported in ppm and are referenced to either TMS or the solvent. MS data were obtained on IonSpec 4.7 Tesla FTMS.
Compound **6**: A solution of compound **5** (97.2 mg, 0.195 mmol), compound **1** (53.0 mg, 0.243 mmol) and Et_3N (0.2 mL) in CH_2Cl_2 (10 mL) was stirred at room temperature overnight. After completion, the reaction was concentrated under reduced pressure and purified by flash column chromatography to give compound **6** as a white solid (124.2 mg, 95%). m.p: 158-162 ℃. IR(KBr) ν_{max} 3387, 2921, 2852, 1798, 1678, 1606, 1578, 1535, 1456, 1262, 1104 CM^{-1}. ^1H NMR (400 MHz, Acetone-d_6) δ 10.14 (s, 1H), 8.88 (t, *J* = 9.5 Hz, 1H), 8.16 (d, *J* = 14.7 Hz, 1H), 7.83–7.70 (m, 1H), 7.30 (dd, *J* = 11.3, 7.4 Hz, 1H), 7.23 (d, *J* = 7.7 Hz, 2H), 7.19–7.13 (m, 1H), 7.13–7.05 (m, 2H), 7.00 (dd, *J* = 9.7, 5.7 Hz, 1H), 6.64 (dd, *J* = 8.5, 5.5 Hz, 1H), 6.59–6.50 (m, 1H), 6.05 (s, 1H), 5.42 (s, 1H), 3.94 (dd, *J* = 19.9, 9.1 Hz, 2H), 3.77 (t, *J* = 13.3 Hz, 1H), 2.92–2.34 (m, 6H), 2.22 (ddd, *J* = 30.4, 19.0, 13.6 Hz, 5H), 2.10 (d, *J* = 6.8 Hz, 1H), 2.03–1.81 (m, 3H), 1.75 (s, 3H), 1.65 (dd, *J* = 13.2, 8.8 Hz, 1H), 1.59–1.22 (m, 8H), 1.17 (d, *J* = 9.5 Hz, 2H), 0.90 (s, 3H); ^{13}C NMR (100 MHz, Acetone-d_6) δ 186.51, 183.23, 172.07, 162.71, 156.21, 153.10, 148.33, 140.42, 139.80, 138.39, 132.02, 128.75, 126.76, 118.73, 116.14, 113.59, 113.13, 98.87, 96.24, 81.58, 80.05, 61.18, 60.65, 58.34, 57.40, 50.53, 45.75, 42.96, 41.79, 40.97, 40.60, 40.06, 39.53, 38.08, 34.26, 31.93, 30.69, 28.26, 27.85, 27.69, 26.52, 25.43, 25.35, 23.98, 23.14, 14.56. MS (ESI) m/z: 670 $(M)^+$. HRMS calcd for $C_{42}H_{46}N_4O_4$ $[M]^+$ 670.35191, found 670.35211.

*General Procedure for Synthesis of **7a-c**:*
A solution of $[Pd(MeCN)_2Cl_2]$ (44.1 mg, 0.170 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise with stirring to a solution of compound **6** (110.0 mg, 0.164 mmol) in anhydrous CH_2Cl_2 (10 mL). After completion, the solution was concentrated, filtered and washed with CH_2Cl_2 to give compound **7a** (67.9 mg, 51%). m.p: 260-265 ℃. IR (KBr) ν_{max} 3369, 2927, 2851, 1728, 1611, 1537, 1451, 1410, 1277, 1106 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.14 (s, 1H), 8.88 (t, *J* = 9.5 Hz, 1H), 8.16 (d, *J* = 14.7 Hz, 1H), 7.83–7.70 (m, 1H), 7.30 (dd, *J* = 11.3, 7.4 Hz, 1H), 7.23 (d, *J* = 7.7 Hz, 2H), 7.19–7.13 (m, 1H), 7.13–7.05 (m, 2H), 7.00 (dd, *J* = 9.7, 5.7 Hz, 1H), 6.64 (dd, *J* = 8.5, 5.5 Hz, 1H), 6.59–6.50 (m, 1H), 6.05 (s, 1H), 5.42 (s, 1H), 3.94 (dd, *J* = 19.9, 9.1 Hz, 2H), 3.77 (t, *J* = 13.3 Hz, 1H), 2.92–2.34 (m, 6H), 2.22 (ddd, *J* = 30.4, 19.0, 13.6 Hz, 5H), 2.10 (d, *J* = 6.8 Hz, 1H), 2.03–1.81 (m, 3H), 1.75 (s, 3H), 1.65 (dd, *J* = 13.2, 8.8 Hz, 1H), 1.59–1.22 (m, 8H), 1.17 (d, *J* = 9.5 Hz, 2H), 0.90 (s, 3H). HRMS Calcd for $C_{42}H_{46}Cl_2N_4O_4Pd$ $[M]^+$ 846.1913, found 846.19111.

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24. Molecular-docking: Estradiol and compound **6** were docked into the into the three-dimensional structure of ERα/β-LBD with the AutoDock software package (version 4.2)[25]. Crystallographic coordinates of small molecules were created by Biochemoffice. The crystal structures of ERα/β-LBD (PDB code: 2YAT, 2OUZ) were obtained from the Protein Data Bank (PDB) and all water molecules were removed. Preparations of all ligands and the protein were performed with AutoDockTools (ADT). A docking cube with the edge of 22.5 Å, 24 Å, 21 Å in X, Y, Z dimension respectively (a grid spacing of 0.375 Å), which encompassed the whole active site, was used throughout docking. On the basis of the Lamarckian genetic algorithm (LGA), 100 runs were performed for each ligand with 500 individuals in the population. The maximum numbers of energy evaluations and of generations were 2.5e7 and 2.7e4, respectively. Other parameters were set as default. The resulting docking solutions were subsequently clustered with a root-mean square deviation (rmsd) tolerance of 2.0 Å and were ranked by binding energy values.
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Design, Synthesis and Biological Evaluation of Novel Estrogen-derived Steroidal Metal Complexes

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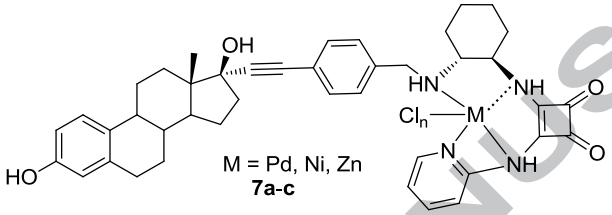

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