EurJOC
European Journal of Organic Chemistry

A Journal of
ChemPubSoc Europe

Accepted Article

Title: Synthesis, cytotoxicity evaluation in human cell lines and in vitro DNA interaction of a hetero arylidene-9(10H)-anthrone

Authors: Paula S. Branco, Daniela Peixoto, Margarida Figueiredo, Gabriela Malta, Catarina Roma-Rodrigues, Pedro Viana Batista, Alexandra R. Fernandes, Sónia Barroso, Ana Luisa Carvalho, Carlos A. M. Afonso, and Luísa Maria Ferreira

To be cited as: *Eur. J. Org. Chem.* 10.1002/ejoc.201701500

Link to VoR: http://dx.doi.org/10.1002/ejoc.201701500

Supported by
ACES Asian Chemical Editorial Society

WILEY-VCH

10.1002/ejoc.201701500

WILEY-VCH

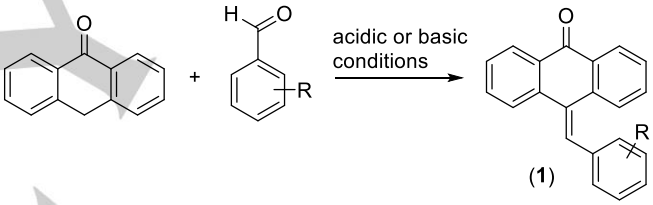
COMMUNICATION

Synthesis, cytotoxicity evaluation in human cell lines and *in vitro* DNA interaction of a hetero arylidene-9(10*H*)-anthrone

Daniela Peixoto,[†] Margarida Figueiredo,[†] Gabriela Malta,[†] Catarina Roma-Rodrigues,[‡] Pedro V. Baptista,[‡] Alexandra R. Fernandes,[‡] Sónia Barroso,[‡‡] Ana Luísa Carvalho,[‡‡] Carlos A. M. Afonso,[§] Luisa M. Ferreira,[*,†] and Paula S. Branco[*,†]

Abstract: A new and never yet reported hetero arylidene-9(10*H*)-anthrone structure (**4**) was unexpectedly isolated on reaction of 1,2-dimethyl-3-ethylimidazolium iodide (**2**) and 9-anthracenecarboxaldehyde (**3**) under basic conditions. Its structure was unequivocally attributed by X-ray crystallography. No cytotoxicity in human healthy fibroblasts and in two different cancer cell lines was observed indicating its applicability in biological systems. Compound **4** interacts with CT-DNA by intercalation between the adjacent base pairs of DNA with a high binding affinity (K_b = 2.0(± 0.20) x 10^5 M^{-1}) which is 10x higher than that described for doxorubicin (K_b = 3.2 (±0.23) × 10^4 M^{-1}). Furthermore, compound **4** quenches the fluorescence emission of GelRed-CT-DNA system with a quenching constant (K_{SV}) of 3.3(±0.3) x 10^3 M^{-1} calculated by the Stern-Volmer equation.

Anthraquinones, whose principal structural features is a tricyclic planar ring system are a group of functionally diverse aromatic compounds displaying a wide range of important pharmaceutical properties.[1] Although historically employed as a natural dye the discovery of various anthraquinone derivatives with medicinal value such as anticancer, antibacterial, anti-inflammatory, antioxidant, antidiabetic, antiviral among others make this scaffold a promising candidate to be studied.[1b, 1c] Doxorubicin,[1b] dauno-rubicin and carminomycin[1c] are successful examples of anticancer agents resulting from their good DNA-intercalating action in result of the flat aromatic anthraquinone core. The anthrone derivatives, 10-substituted benzylidene anthrones,[2] also show good anti-tumor activities with some compounds acting as inhibitors of tubulin polymerization.[2b, 3] Here, the three rings of the anthrone system are not co-planar because of steric interactions with the benzylidene group.[4] The 10-substituted benzylidene anthrone (1) may be prepared with moderate yields by reaction of anthrone and substituted benzaldehydes under acidic (gaseous HCl) or basic (pyridine/piperidine) conditions (Scheme 1).[2a, 2b] Notwithstanding the extensive work on imidazolium-based ionic liquids (IL), only but a few references exist concerning the reactivity of these azolium salts when functionalized at the C2-position.[5] In the presence of a base the *N*-heterocyclic olefin (NHO)[6] those formed act as a nucleophile reacting with aldehydes,[7] with CO_2,[8] as catalyst in the trans-esterification reaction,[6] is susceptible to alkylation [9] or, can be involved in coupling reactions.[10]



Scheme 1. Synthesis of 10-substituted benzylidene anthrones (**1**).

Recently,[11] we have disclosed an unusual reactivity of 1,2-dimethyl-3-ethylimidazolium salt (**2**). We have observed that on reaction with aldehydes, an unpredicted result with the oxidation to the corresponding carboxylic acid took place. Here we present our unexpected and singular result, obtained when on the above described reaction the substrate is the 9-anthracenecarboxaldehyde.

Applying the reaction conditions described previously,[11] **2**, cesium carbonate and 9-anthracenecarboxaldehyde (**3a**) in dry THF, the hetero arylidene-9(10*H*)-anthrone (**4**) was obtained after 72h of reaction, in 70% yield (Table 1, entry 2). The structural assignment of **4** (see SI) was unequivocally confirmed by single-crystal X-ray crystallography (Figure 1). Under the reactions conditions an isomeric structure **4a** was isolated, which is interconverted to **4** in DMSO solution, presents the chemical shifts for the imidazolium part slightly shielding in result of the asymmetric boat conformation of the anthrone system. The central ring of the anthrone unit is not co-planar (observed in other substituted anthrones.[4]) with the two outer benzene rings due to steric interactions between the anthrone moiety and the imidazolium group. The outer benzene rings form a dihedral angle of 20.7(3)˚ and atoms C2, C7 C9 and C14 in the central ring are

[†] Dr. D. Peixoto, G.Malta, M. Figueiredo, Prof. L. M. Ferreira, Prof. P. S. Branco
LAQV-REQUIMTE, DQ, 2829-516, Caparica, Portugal
Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa
2829-516, Caparica, Portugal
E-mail: lpf@fact.unl.pt
(https://www.dq.fct.unl.pt/pessoas/docentes/luisa-maria-da-silva-pinto-ferreira)
paula.branco@fct.unl.pt
(https://www.dq.fct.unl.pt/pessoas/docentes/paula-cristina-de-serio-branco)
[‡] Dr. C. Roma-Rodrigues, Prof. A. R. Fernandes, Prof. P. V. Batista
UCIBIO-REQUIMTE, DCV
Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa
2829-516, Caparica, Portugal
[‡‡] Dr. S. Barroso, Dr. A. L. Carvalho
UCIBIO-REQUIMTE, DQ
Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa
2829-516, Caparica, Portugal
[§] Prof. C. A. M. Afonso
iMed.ULisboa
Faculdade de Farmácia, Universidade de Lisboa
Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

Supporting information for this article is given via a link at the end of the document.

co-planar (R_{ms} = 0.0138 Å), with atoms C1 and C8 deviating from the plane by 0.139(10) and 0.297(10) Å, respectively (Figure 1).

To elucidate the mechanism for the formation of **4**, other 10-substituted 9-anthracenecarboxaldehyde derivatives (**3b**-**3d**) were subject to the reaction conditions (Table 1) and, surprisingly, compound **4** was also isolated upon reaction with 10-chloro-9-anthracenecarboxaldehyde (**3b**) and 9,10-anthracenedicarboxaldehyde (**3c**) in 90% and 37% yields respectively (entries 10 and 16) but absent upon reaction with 10-methyl-9-anthracenecarboxaldehyde (**3d**) (entry 20). To elucidate the mechanism of the reaction it was crucial to identify minor compounds isolated in the ethereal fraction from the washing of the crude reaction mixture. Common to all reaction is the formation of anthraquinone (**5**), although the amount is dependent on the reaction time and substrate. We postulate its formation through fragmentations of the endoperoxides (**6a-c**) (Scheme 2)

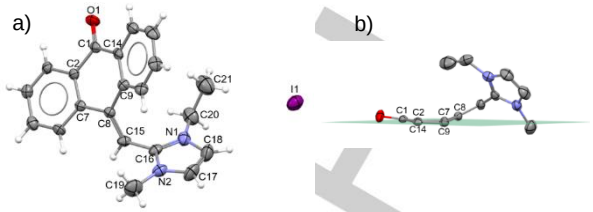
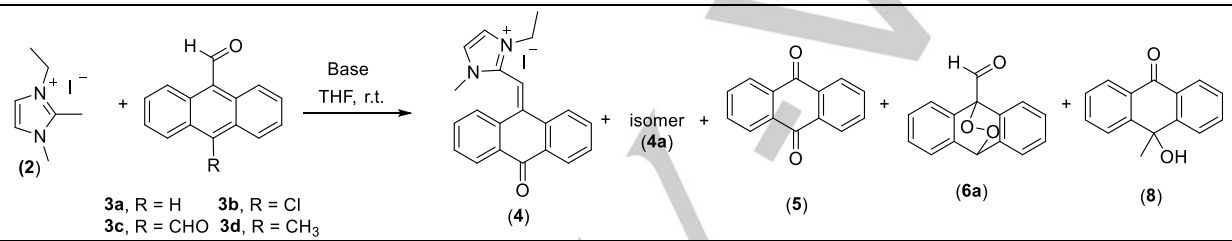


Figure 1. a) MERCURY diagram of compound **4**, using 30% probability level ellipsoids; b) the asymmetric boat conformation of the central ring in the anthrone unit. Hydrogen atoms and the outer aromatic rings are omitted for clarity

Table 1. Reaction conditions of **2** and **3**.



3a, R = H **3b**, R = Cl
3c, R = CHO **3d**, R = CH_3

Entry	9-anthracene aldehyde (3) (1 equiv)	Base	2 (equiv)	Time (h)	Recovered 3 (%)	4 (%)	4a (%)	5 (%)	6a (%)	8 (%)
1a	3a	Cs_2CO_3 (1.2 equiv)	0	48	20	-	-	3.2	19.6	-
1b		NEt_3 (1.2 equiv)	0	48	100	-	-	-	-	-
2		Cs_2CO_3 (1.2 equiv)	1	72		70	-	13	6	-
3[a)]		Cs_2CO_3 (1.2 equiv)	1	72	30	20	-	45	3.2	-
4[b)]		Cs_2CO_3 (1.2 equiv)	1	72	88	5.2		5	vestg.	
5		NaH (1.2 equiv)	1	72	-	vestg.	-	46	-	-
6		NaOH (1.2 equiv)	1	120	-	-	72	27	-	-
7		NEt_3(1.2 equiv)	1	120	90	-	-	-	6	-
8		Piperidine (1.2 equiv)	1	72	79	-	-	5	10	-
9	3b	Cs_2CO_3 (1.2 equiv)	0	48	40	-	-	15	-	-
10		Cs_2CO_3 (1.2 equiv)	1	72	5	90	-	5	-	-
11		NaH (1.2 equiv)	1	72	23	8.8	-	12	-	-
12		NaOH (1.2 equiv)	1	120	-	82	-	10	-	-
13		NEt_3 (1.2 equiv)	1	120	99	-	-	-	-	-
14		Piperidine (1.2 equiv)	1	72	89	-	-	3.5	-	-
15	3c	Cs_2CO_3 (1.2 equiv)	0	48	75	-	-	5	-	-
16		Cs_2CO_3 (1.2 equiv)	1	72	10	37	-	40	-	-
17		Cs_2CO_3 (2.4 equiv)	2	168	vestg	-	8	60	-	-
18[b)]		Cs_2CO_3 (1.2 equiv)	1	72	35	9	-	14.6	-	-
19	3d	Cs_2CO_3 (1.2 equiv)	0	48	35	-	-	22	-	32.7
20		Cs_2CO_3 (1.2 equiv)	1	168	25	-	-	45	-	20

a) THF saturated with O_2; b) THF degassed

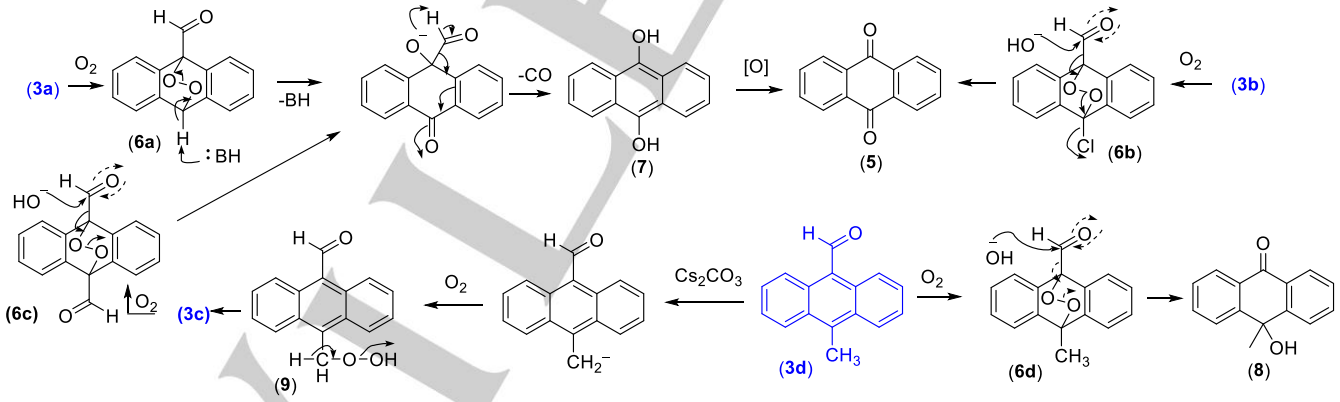
under basic conditions formed due to the presence of residual oxygen on the solvent. Bauch et al. have recently[12] reported that the decomposition of anthracene endoperoxides proceeds via various intermediates, as for example the 9,10-dihydroxyanthracene (**7**). We also proposed the formation of intermediate **7**, which is subsequently oxidized to **5** (Scheme 2). We isolate compound **6a** and its structure was attributed by ^{1}H NMR. As expected, the mass spectra only shows the [M-

10.1002/ejoc.201701500

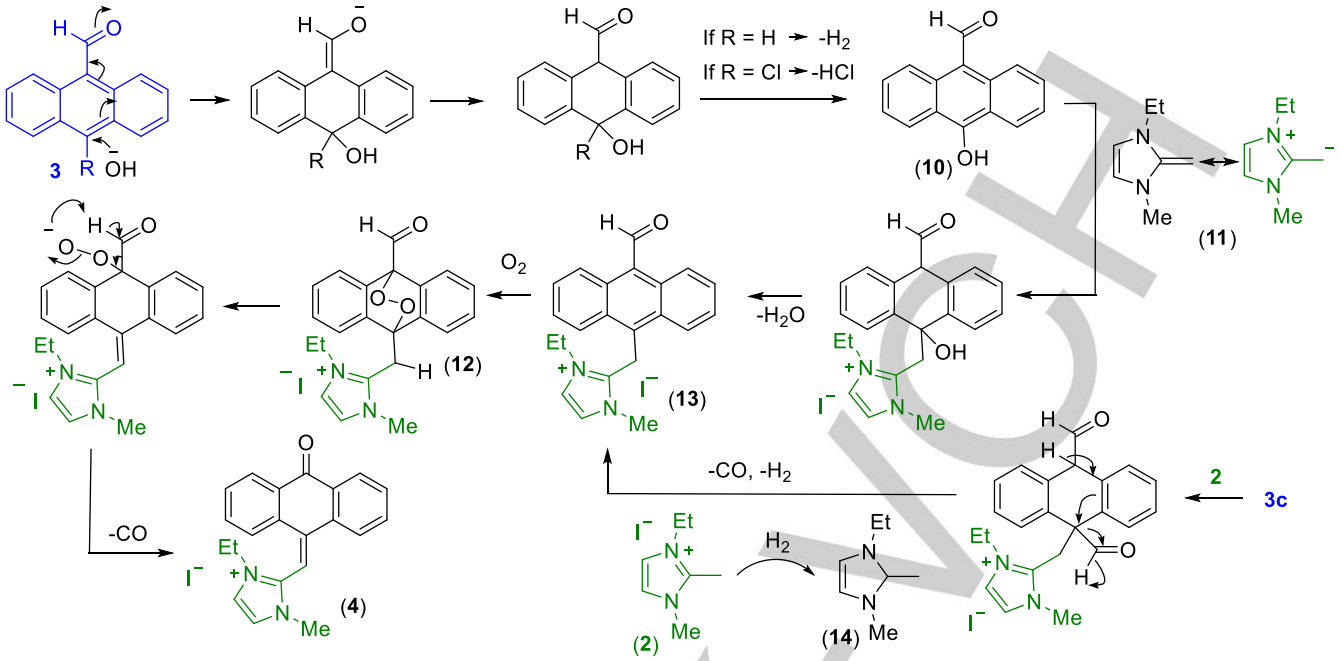
32]$^+$ ion. The formation of the endoperoxides intermediates justifies that upon reaction with **3d** the anthraquinone **5** and the 10-hydroxy-10-methylanthracen-9(10*H*)-one (**8**) (Scheme 2) were the major products isolated (entries 19 and 20). Since in the reaction with **3d**, the dialdehyde **3c** was identified in residual amount by GC-MS, we postulate the formation of the hydroperoxide **9** and endoperoxide **6d** as intermediates to **5** and **8** respectively. To clarify the formation of **4** and **5** additional experiments were conducted, namely in the absence or presence of O_2 and with other bases besides Cs_2CO_3 such as, NEt_3, NaOH or NaH. Only nucleophilic bases, such as NaOH (entries 6 and 12) or Cs_2CO_3 (entries 2, 10 and 16) allowed the formation of **4**, and small amounts were observed with NaH (entry 5 and 11). With NEt_3 as base, the starting materials **3a** was recovered and **6a** observed in 6% yield (entries 7 and 13), which reinforces the need of a stronger base to remove the proton from **6a** (Scheme 2).No reaction was observed between **5** and **2** in the presence of Cs_2CO_3 in THF, which led to exclude **5** as the intermediate for the formation of **4**. We have also observed the release of CO in the reaction medium and a decrease of the yield, from 70% to 5.2% when the reaction was performed in absence of O_2 (entries 2 vs 4) which prompted us to present the mechanism in Scheme 3. Since only nucleophilic bases allowed the formation of **4**, we proposed a 1,6 conjugated addition of the base with formation of 10-hydroxyanthracene-9-carbaldehyde (**10**). This intermediate upon addition of the NHO (**11**) and after water elimination followed by reaction with O_2 gave the endoperoxide **12** through the anthracene derivative intermediate **13**.The CO release (observed qualitatively in higher amount after 72h by GC-TCD), led to the formation of **4**. A higher amount of CO was detected when the reaction was performed with **3c.** The reduced specie (**14**) previously observed by us [11], was here identified which supports the proposed mechanism involving the oxidation to **13**. Although the reaction was performed with dry THF, no particular caution was taken to exclude the contact with air, with the concomitant presence of oxygen with time. This, and the presence of moisture, can explain the formation of **4** in small amounts when NaH was used as base (entry 11). We would expected, taking in consideration the above proposed mechanism that the yield for the formation of **4** would increase in the presence of an O_2 atmosphere and decrease in the exclusion of O_2. Indeed, vestigial amounts were observed in the absence of O_2 but with an O_2 atmosphere, the parallel reaction of the **3** with O_2 (scheme 3) lead to the increase amount of **5** (entries 2 vs 3).

Compound **4** cytotoxicity was assessed by the MTS assay on representative human cancer cell lines: ovarian carcinoma (A2780) and colorectal carcinoma (HCT116), and compared to normal human primary fibroblasts (see SI Figure S1). No impairment of cell viability is observed after the incubation of the compound in all cell lines for the low micromolar range. Compound **4** shows no toxicity to human fibroblasts contrary to doxorubicin.[13] This demonstrates that compound **4** may be used in biological applications due to the lack of cytotoxicity in those human cell lines. Also, exposure of A2780 cells for 6 h to a high concentration of compound **4** (1000 µM) or to 250 µM (IC_{50}) for 48 h does not induce reactive oxygen species (ROS) (SI Figure S2).

The UV-visible absorption spectra of compound **4** after a 24 h incubation period in Tris-HCl 0.5 mM pH 7.0 with 50 mM NaCl may be observed in SI.



Scheme 2. Proposed mechanism for the formation of **5** and **8**.



Scheme 3. Proposed mechanism for the formation of **4**.

Since DNA is one of the cellular targets of therapeutic anthraquinones (e.g. doxorubicin), whose flat aromatic anthraquinone core allows intercalation between the DNA bases,[1b, 1c] we evaluated the interaction *in vitro* between compound **4** and CT-DNA. Following incubation of CT-DNA (15 µM) with increasing concentrations of compound **4**, UV-spectra were acquired (Figure 2). The absorption intensity of CT-DNA decreases (hypochromism) with increasing concentration of compound, a characteristic of intercalation usually attributed to the interaction between the electronic states of anthraquinones and those of DNA nucleobases. Corroborating this hypothesis, the hypochromism of CT-DNA in the presence of compound **4** is still observed under higher ionic strength conditions (SI Figure S3).

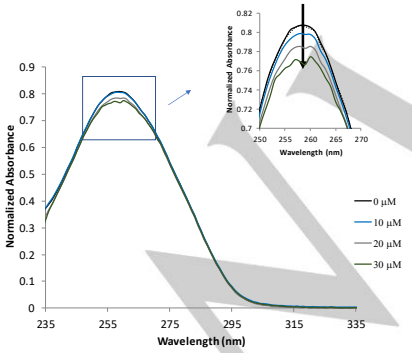


Figure 2. Absorption spectra of CT-DNA (15 µM) in the presence of increasing concentrations of compound **4** (0-30 µM). Inset: maximum absorbance peak hypochromism due to the increase of compound **4** concentrations – indicated by the black arrow.

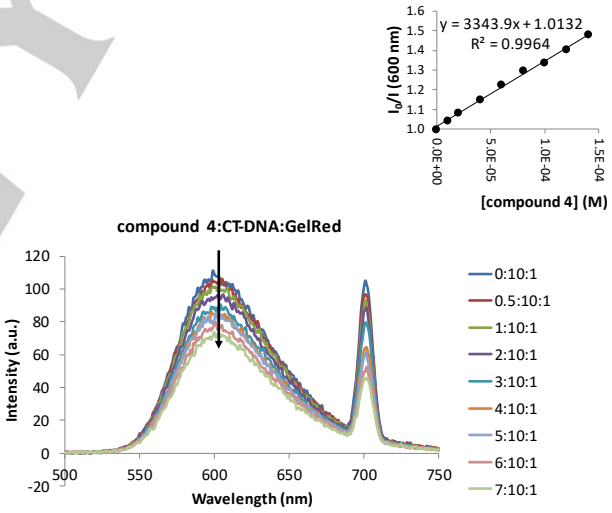


Figure 3. Absorption spectra of the competitive reaction between different ratios of compound **4** and GelRed bonded to CT-DNA (different lines - ratios compound **4**: CT-DNA: GelRed), GelRed = 20 µM and CT-DNA = 200 µM Tris-HCl 0.5 mM pH 7.0 with NaCl 50 mM. The black arrow indicates the quenching effect observed with increasing concentrations of compound **4**. Inset: plot of (I0 /I) versus [Compound **4**] to calculate the quenching constant.

This is consistent with the combination of compound **4** π electrons and π electrons of DNA nucleobases with the consequent decrease of the energy level of the π-π* electron transition.[14] The calculated intrinsic binding constant K_b is 2.0(± 0.20) x 10^5 M^{-1}. The K_b values described in the literature for classical intercalators (e.g. ethidium bromide–DNA, 7 × 10^7 M^{-1}),[15] are at least 100× higher than that of compound **4**.

However, the intrinsic binding constant is 10x higher than that described for doxorubicin (K_b = 3.2 (±0.23) × 10^4 M^{-1}).[16]

The binding of compound **4** to CT-DNA as a competitive intercalative binding probe with GelRed[17] was further analyzed. In the competitive binding experiments, GelRed was first incubated with CT-DNA for 30 min to ensure sufficient binding sites between GelRed and DNA (concentration ratio was set at [GelRed]/[CT-DNA] = 1:10). The emission spectra of GelRed–CT-DNA system in the absence and presence of increasing concentrations of compound **4** are shown in Figure 3. As expected, when excited at 350 nm, the GelRed–CT-DNA system presented a characteristic fluorescence emission at around 590 nm, indicating intercalation of GelRed within the adjacent nucleobases. The presence of compound **4** considerably quenched the GelRed's fluorescence emission (Figure 3 – indicated by the black arrow) with no observed saturation state until a [compound **4**]/[GelRed] ratio of 7. The quenching constant (K_{SV}) for compound **4** bound to GelRed-DNA system was determined 3.3(±0.3) x 10^3 M^{-1} by Stern-Volmer equation.

Circular dichroism (CD) was also used to clarify the nature of the interactions between compound **4** and CT-DNA, i.e. electrostatic interaction or minor groove binding or intercalation.[18] Results showed a modification on both positive and negative bands of the B-form of CT-DNA when compound **4** is added (SI Figure S4), which corroborates that compound **4** intercalates within adjacent nucleobases.

Here we report for the first time an unusual hetero arylidene-9(10*H*)-anthrone salt (**4**). Its structure was unequivocally attributed by X-ray crystallography. A detailed mechanistic study was carried out to provide insights to the unexpected reactivity of the imidazolium salt and its oxidative role. Alongside decarbonylation and the involvement of endoperoxide species are proposed in the pathway for the formation of the anthrone derivative **4**. Compound **4** showed no cytotoxicity in human normal healthy fibroblasts or in two different cancer cell models, indicating it might be suitable for several applications in biological systems. Compound **4** strongly interacts with CT-DNA by intercalation (K_b = 2.0(± 0.20) x 10^5 M^{-1}), and is able to displace GelRed from the DNA strands, thus quenching fluorescence of GelRed-CT-DNA system (K_{SV}=3.3 ±0.3 x 10^3 M^{-1}).

Acknowledgements

This work was supported by the LAQV, which is financed by national funds from FCT/MEC(UID/QUI/50006/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145- 710-FEDER-007265), and by UCIBIO by national funds from FCT/MEC (UID/Multi/04378/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER-007728). The National NMR Facility and the SCXRD facility are supported by Fundação para a Ciência e Tecnologia (RECI/BBB-BQB/0230/2012 and RECI/BBB-BEP/0124/2012, respectively). We thank Dr. R. Pandian for the crystallographic data collection. We acknowledge the Laboratório de Análises REQUIMTE for the technical support for the mass spectrometry analyses.

Keywords: imidazolium salt • anthracenecarboxaldehyde • arylidene anthrone • decarbonylation • DNA intercalation

[1] a) E. M. Malik, C. E. Muller, *Med. Res. Rev.* **2016**, *36*, 705-748; b) M. N. Preobrazhenskaya, A. E. Shchekotikhin, A. A. Shtil, H.-S. Huang, *J. Med. Sci.* **2006**, 26001; c) H. Hussain, A. Al-Harrasi, A. Al-Rawahi, I. R. Green, R. Csuk, I. Ahmed, A. Shah, G. Abbas, N. U. Rehman, R. Ullah, *Expert Opin. Ther. Patents* **2015**, *25*, 1053-1064.

[2] a) W. X. Hu, W. Zhou, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 621-622; b) H. Prinz, Y. Ishii, T. Hirano, T. Stoiber, J. A. C. Gomez, P. Schmidt, H. Dussmann, A. M. Burger, J. H. M. Prehn, E. G. Gunther, E. Unger, K. Umezawa, *J. Med. Chem.* **2003**, *46*, 3382-3394; c) H. F. Li, M. D. H. Hassona, N. A. Lack, P. Axerio-Cilies, E. Leblanc, P. Tavassoli, N. Kanaan, K. Frewin, K. Singh, H. Adomat, K. J. Bohm, H. Prinz, E. T. Guns, P. S. Rennie, A. Cherkasov, *Mol. Cancer Ther.* **2013**, *12*, 2425-2435.

[3]K. D. Paull, C. M. Lin, L. Malspeis, E. Hamel, *Cancer Res.* **1992**, *52*, 3892-3900.

[4]W. Zhou, W. X. Hu, C. N. Xia, M. Q. Chen, *Acta Crystallogr. Sect. E.-Struct Rep. Online* **2005**, *61*, O3170-O3171.

[5]B. Wang, L. Qin, T. Mu, Z. Xue, G. Gao, *Chem. Rev* **2017**, 7113–7131.

[6]M. Blumel, J. M. Noy, D. Enders, M. H. Stenzel, T. V. Nguyen, *Org. Lett.* **2016**, *18*, 2208-2211.

[7]C. G. Fortuna, V. Barresi, C. Bonaccorso, G. Consiglio, S. Failla, A. Trovato-Salinaro, G. Musumarra, *Eur. J. Med. Chem.* **2012**, *47*, 221-227.

[8]Y. B. Wang, Y. M. Wang, W. Z. Zhang, X. B. Lu, *J. Am. Chem. Soc.* **2013**, *135*, 11996-12003.

[9]A. Dumrath, X. F. Wu, H. Neumann, A. Spannenberg, R. Jackstell, M. Beller, *Angew. Chem. Int. Ed.* **2010**, *49*, 8988-8992.

[10]M. Schedler, N. E. Wurz, C. G. Daniliuc, F. Glorius, *Org. Lett.* **2014**, *16*, 3134-3137.

[11]D. Peixoto, M. Figueiredo, M. B. Gawande, M. C. Corvo, G. Vanhoenacker, C. A. M. Afonso, L. M. Ferreira, P. S. Branco, *Journal of Organic Chemistry* **2017**, *82*, 6232-6241.

[12]M. Bauch, M. Klaper, T. Linker, *J. Phys. Org. Chem.* **2017**, *30*.

[13]V. Kuete, L. K. Omosa, V. R. S. Tala, J. O. Midiwo, A. T. Mbaveng, S. Swaleh, O. Karaosmanoglu, H. Sivas, *BMC Pharmacol. Toxicol.* **2016**, *17*.

[14] A. A. Ensafi, R. Hajian, S. Ebrahimi, *J. Braz. Chem. Soc.* **2009**, *20*, 266-276.

[15] M. J. Waring, *J. Mol. Biol.* **1965**, *13*, 269-&.

[16] R. Hajian, N. Shams, M. Mohagheghian, *J. Braz. Chem. Soc.* **2009**, *20*, 1399-1405.

[17] M. Liu, H. M. Zhao, S. Chen, H. T. Yu, Y. B. Zhang, X. Quan, *Chem. Commun.* **2011**, *47*, 7749-7751.

[18] S. U. Rehman, T. Sarwar, M. A. Husain, H. M. Ishqi, M. Tabish, *Arch. Biochem. Biophys*. **2015**, *576*, 49-60.

10.1002/ejoc.201701500

WILEY-VCH

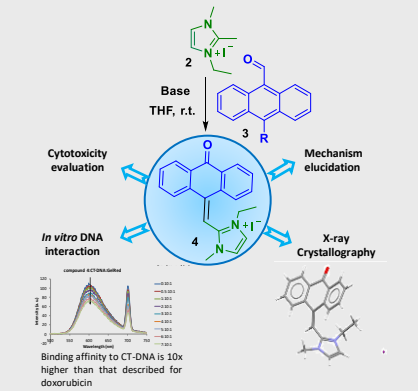
Entry for the Table of Contents (Please choose one layout)

Layout 1:

COMMUNICATION

Synthesis, cytotoxicity evaluation in human cells lines and in vitro DNA interaction of a hetero arylidene-9(10*H*)-anthrone

Daniela Peixoto,[†] Margarida Figueiredo,[†] Gabriela Malta,[†] Catarina Roma-Rodrigues,[‡] Pedro V. Baptista,[‡] Alexandra R. Fernandes,[‡] Sónia Barroso,[‡] Carlos A. M. Afonso,[§] Luisa M. Ferreira,*,[†] and Paula S. Branco*,[†]



A hetero arylidene-9(10H)-anthrone structure (4) was unexpectedly isolated on reaction of imidazolium salt (2) and 9-anthracenecarboxaldehyde (3) which revealed no cytotoxicity and was able to interact with CT-DNA by intercalation. Its structure was unequivocally attributed by X-ray crystallography. Decarbonylation and the involvement of endoperoxide species are proposed on mechanistic studies.

Key Topic: N-Heterocyclic olefin reactivity

*Author(s), Corresponding Author(s)**

Page No. – Page No.

Title