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Synthesis and biological evaluation of 2,6-di(furan-3-yl)anthracene-9, 10-dione as an inhibitor of human protein kinase CK2

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Protein kinase CK2 is an emerging target for the therapeutic intervention in human diseases, in particular in cancer. Inhibitors of this enzyme are at current in clinical trials indicating its druggability. Here we report on the synthesis of two derivatives of 2,6-diaryl-anthracene-9,10-dione, one of them, 2,6-di(furan-3-yl)anthracene-9,10-dione **(3)**, turned out to be active towards CK2, and ATP competitive with an IC_{50} value of 2.35 μM and a K_i value of 1.26 μM. Molecular modeling studies were performed to explain the binding affinity of compound **3** in comparison to emodin. These indicated that unlike emodin, compound **3** was not able to perform a hydrogen bond with Lys68, although the compound fits well in the active site of human CK2α, which explains the difference in the measured affinity between those two compounds.

1. Introduction

Casein Kinase 2 (CK2) is an ubiquitous eukaryotic serine/threonine protein kinase, which has a heterotetrameric structure, commonly referred to as the CK2 holoenzyme, composed of two catalytic subunits (α and/or α') and two regulatory subunits (β) (Prowald et al. 1984; Yenice et al. 1994; Faust et al. 1996; Ahmad et al. 2005). The human protein kinase CK2 which was discovered in 1954 by Burnett and Kennedy has a dual co-substrate specificity as it is able to use ATP or GTP as the phosphate group donor (Salvi et al. 2009). The enzyme is constitutively active, either the free catalytic subunit(s) alone (α and/or α') or the heterotetrameric complex of two catalytic subunits attached to a dimer of β subunits, and is supposed to phosphorylate hundreds of protein substrates (Suzuki et al. 2008). CK2 has important roles in different cellular functions, such as signal transduction, DNA repair and gene expression (Meggio and Pinna 2003; Pinna 2003). CK2 enhances cancer phenotype by blocking apoptosis and stimulating cell growth. Thus, inhibition of CK2 can induce the physiological process of apoptosis leading to tumor cell death (Slaton et al. 2004; Ahmad et al. 2008). CK2 is considered to be a dependable therapeutic target for the treatment of different types of cancer (Trembley et al. 2010). The first known protein kinase inhibitor was described in the early 80's (Hidaka et al. 1980), and since then, a large number of compounds has been developed as kinase inhibitors including CK2 inhibitors (Cozza et al. 2010; Cozza et al. 2012, Cozza et al. 2013). However, the first patent on CK2 inhibiting compounds was granted as late as 2004 (Cozza et al. 2012). Most of the CK2 inhibitors known today contain a heterocyclic backbone which fits into the active site of the CK2α and competes with the ATP (Cozza et al. 2009). Several review articles were published focusing on CK2 and the increasing number of inhibitors (Cozza et al. 2010; Cozza et al. 2013; Venerando et al. 2014), but only one compound (CX–4945) is known to be in clinical trials phase II as CK2 inhibitor so far. Among the published inhibitors is emodin (Fig. 1) which was described as an active CK2 inhibitor with a K_i value of 1.5 μM (Yim et al. 1999; Sarno et al. 2002; Raaf et al. 2008). Niefind et al. previously developed a crystal structure of emodin in complex with the catalytic subunit of protein kinase CK2 (PDB: 3C13). In this crystal structure the authors showed a hydrogen bond between the hydroxyl group in position 3 of emodin and the Lys68 as well as a π-π interaction between the ring A of emodin and the aromatic ring in Phe113. Since it is not always possible to increase the activity of starting hits, although it is the aim of all researchers in this field, new results will be important for the developent of better inhibitors.

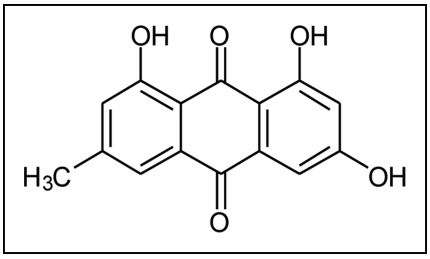


Fig. 1: Structure of the known CK2 inhibitor emodin (Yim et al. 1999).

In this work the structure of emodin was modified by introducing two heterocycles in the emodin backbone with the aim to increase the inhibitory activity. In fact, the introduction of heterocycles to different scaffolds was presented in several CK2 inhibitors (Guillon et al. 2013), and flanked heterocycles or aromatic rings are present in some CK2 inhibitor backbones, namely pyrazolo-triazine derivatives or disubstituted pyrazine (Nie et al. 2007; Suzuki et al. 2008; Fuchi et al. 2012; Cozza et al. 2013), two examples are shown in Fig. 2. Also introduction of a pyrimidine ring in a CX–4945 like compound increased the

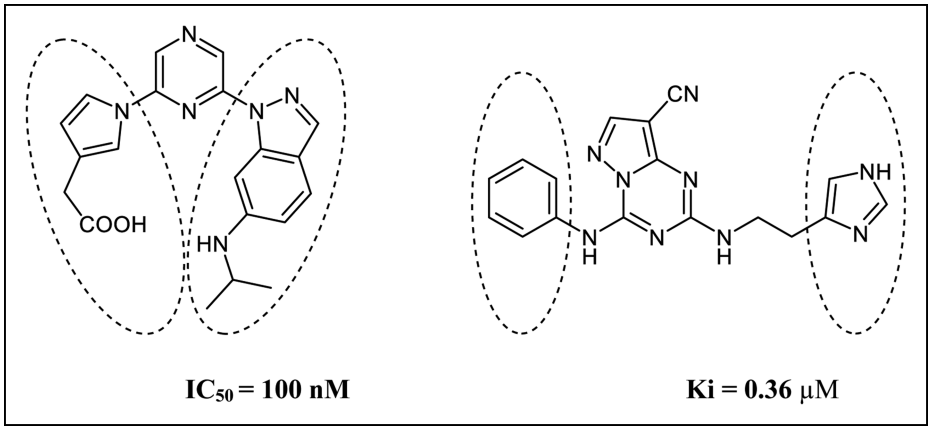


Fig. 2: Structures of the known CK2 inhibitors with flanked aromatic groups (Nie et al. 2007; Fuchi et al. 2012).

selectivity towards CK2 (Cozza et al. 2013). The aim of introducing the heterocycles was to add more hydrogen bonds, which might increase the affinity of the compounds. The main idea was to explore if the furan oxygen (or pyrimidine nitrogen) can form hydrogen bonds with the amino acid residue and might have better orientation than emodin, and also to investigate if the aromatic furan or pyrimidine ring can form π-π interactions instead of the aromatic ring A of emodin. Having a flanked symmetrical compound might also increase the chance of binding in the binding site of this enzyme since we expect that the heterocycle can play a role in binding. Therefore two compounds were synthesized based on the 9,10-anthraquinone backbone bearing two furan or two pyrimidine rings in the 2 and 6 positions and tested for inhibition of recombinant human protein kinase CK2.

2. Investigations, results and discussion

2.1. Synthesis

The Suzuki coupling was applied for the synthesis of 2,6-diaryl-9,10-anthraquinones by transferring the 2,6-dihydroxy-9,10-anthraquinone into its bistriflate. The bistriflate was then reacted with arylboronic acids as described for the synthesis of 2,6-di(furan-2-yl)anthracene-9,10-dione (Gautrot et al. 2007). Furan-3-boronic acid and 5-pyrimidinboronic acid were used to gain two new compounds, namely 2,6-di(furan-3-yl)anthracene-9,10-dione (**3**) and 2,6-di(pyrimidine-5-yl)anthracene-9,10-dione (**4**), and purification was performed by HPLC. The synthesis pathway is presented in the Scheme 1. It is important to note that the yield of the Suzuki reaction was low, particularly for compound **4** (yield less than 5%). Modifications in the reaction conditions, such as reaction temperature and time were performed but did not help much and for that reason the chemical data and biological results were only presented for compound **3** (yield 30 %) which is a new compound, not described before.

2.2. Determination of biological activity

The synthesized compound 2,6 diaryl-9,10-anthraquinone (**3**) was tested for its inhibitory activity towards the human CK2 holoenzyme following a procedure described earlier (Olgen et al. 2007). The synthetic peptide RRRDDDSDDD was used as the substrate, which is reported to be most efficiently phosphorylated by CK2. The purity of the CK2 holoenzyme was superior to 99 %. For initial testing, inhibition was determined relative to the controls at inhibitor concentrations of 10 μM in DMSO as solvent. The reaction with pure solvent without inhibitor was used as negative control and set to 0 % inhibition. Reactions without CK2 were used as positive control and were taken as 100 % inhibition. IC_{50} values were determined by measuring CK2 inhibition at nine different concentrations ranging from 0.001 to 50 μM in appropriate intervals and calculated from the resulting dose–response curve (Gratz et al. 2010). The IC_{50} of compound **3** turned out to be 2.35 μM and for comparison the IC_{50} value of emodin measured as a control was 0.58 μM.

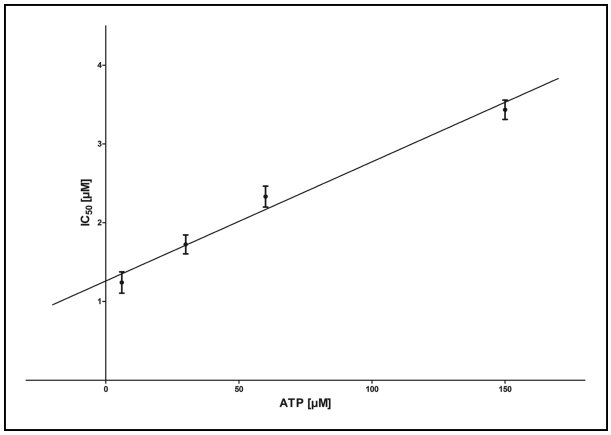
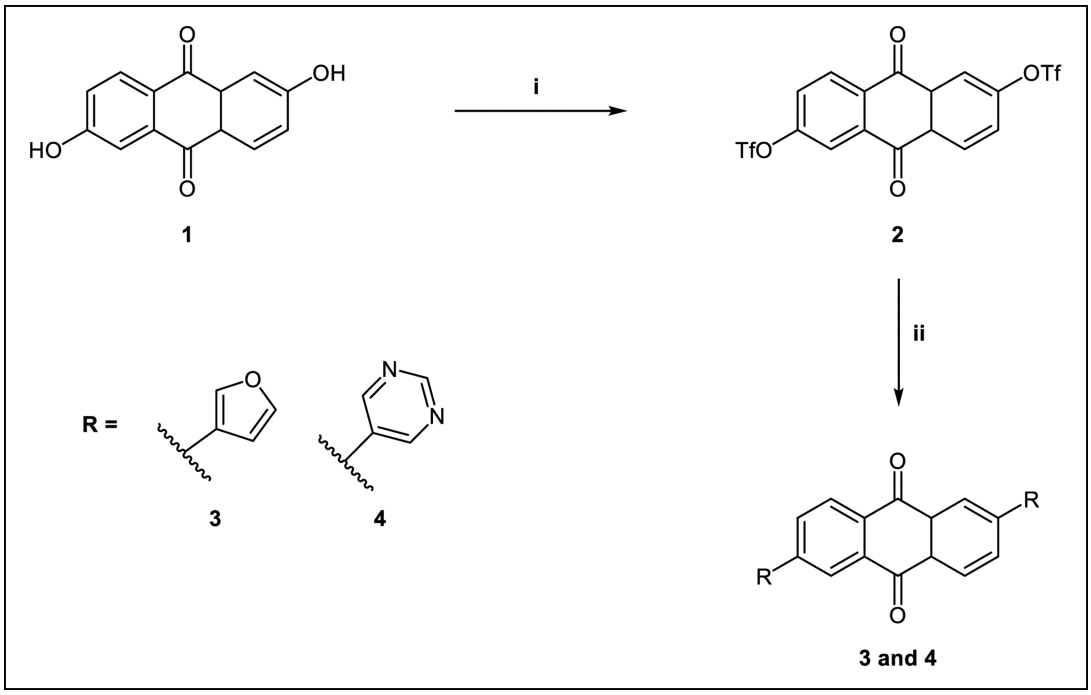


Fig. 3: ATP-competitive inhibition of human CK2 by compound **3**. IC_{50} values were determined using nine different concentration of the inhibitor ranging from 0.001 to 50 μM and plotted against the respective ATP concentrations. Each IC_{50} values was determined 3 times independently. Mean values with corresponding standard deviations are given. The K_i value is defined as the Y-intercept and was calculated to be 1.26 μM (R^2 = 0.9299).

In order to validate the assumed ATP competitive mode of action for compound **3**, IC_{50} values were determined at four different ATP concentrations. IC_{50} values were observed to increase linearly with the ATP concentration. In consequence this kinetic study demonstrated that compound **3** is indeed ATP competitive (Fig. 3).

The initial plan for this project was to prepare more derivatives using different arylboronic acids, but the low yield of compounds obtained thereby and the moderate activity of compound **3** directed the synthesis towards different scaffolds. In order to explain the difference in the IC_{50} values between emodin and compound **3** (2.35 μM versus 0.58 μM), it is important to have a look at the binding mode of compound **3** in the ATP binding site of the enzyme. For that reason, a molecular docking study was performed using the Molecular Operating Environment (MOE) software from Chemical Computing Group (Molecular Operating Environment (MOE)). It became clear from this study that unlike emodin, compound **3** does not undergo direct hydrogen bonding with Lys68, which could be a reason for the reduced binding affinity. Furthermore, as can be seen in Fig. 4b, compound **3** fits well inside the ATP-binding pocket of human CK2,



Scheme 1: Synthesis of 2,6-bisaryl-9,10-anthraquinones (compounds **3** and **4**). i: Trifluoromethanesulfonic anhydride, Py., HCl, ii: arylboronic acid, $Pd(PPh_3)_4$, THF, Na_2CO_3.

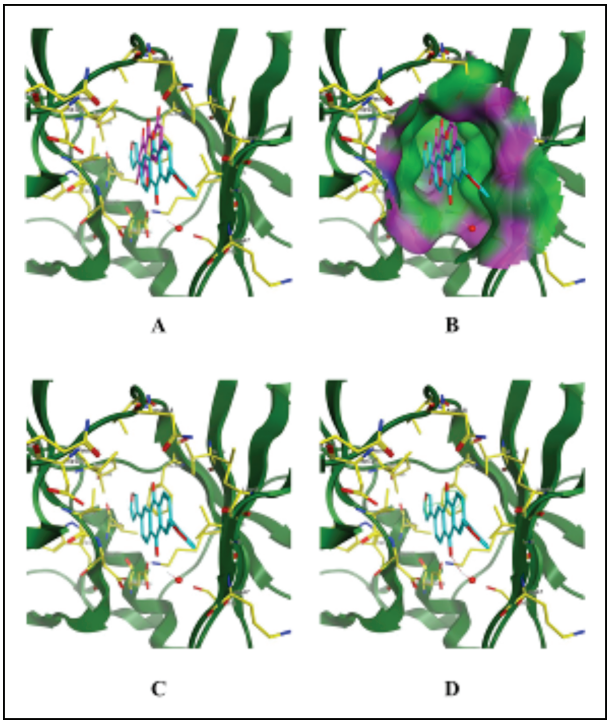


Fig. 4: A) Superimposition of the co-crystallized inhibitor emodin and the docked inhibitor **3**. B) Illustration of the ATP-binding pocket with the two inhibitors. **C)** Predicted binding mode of **3** in the ATP-binding site of human CK2 catalytic subunit. D) Predicted interactions between **3** and the ATP binding site. (pictures were generated by MOE and 3C13 crystal structure was used).

and forms π-π interactions with Phe113, as well as an indirect interaction *via* a water molecule with Ser51 residue which may contribute to the affinity of the compound. Figure 4 gives the binding modes of emodin and compound **3** in the ATP-binding pocket of CK2α, while Fig. 5 compares the 2D interactions of emodin and compound **3** in the ATP-binding pocket of the CK2α. In matter of fact it would have been better to perform the docking before the synthesis, but it is important also to note that the none encouraging simulation results should not always stop the synthesis.

In conclusion, the modifications on the anthraquinone backbone in this work produced a new active CK2 inhibitor with an IC_{50} value in the low micromolar range. In fact the yield of the developed compounds was not high, but indeed the yield of Suzuki reactions might vary according to the conditions such as reaction temperature, catalyst, reaction time and other conditions, and many studies were focusing on the improvement of the yield of such reactions (Borhade and Waghmode 2011). In conclusion, a novel compound was synthesized and tested in a kinetic and modeling study. Although compound **3** is less active than emodin depending on IC_{50} results (Ki for emodin was not determined by us), our results can help to further elucidate the topography of the active site of the enzyme and appear to be the basis for further studies to optimize the activity by preparing other derivatives.

3. Experimental

3.1. Materials and methods

Melting points were determined on a Mettler Toledo apparatus (MP50 Melting Point System), and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on an Agilent apparatus DD2 400 MHz, DD2 600 MHz, using deuterated chloroform or deuterated dimethylsulfoxide as solvents. The chemical shifts are expressed as δ values in ppm against TMS as the internal standard. Purities and molecular masses of compounds were determined by Bruker, Daltonics micro TOF-QII, IR spectra were recorded using Shimadzu, MIRacle 10 Single Reflection ATR Accessory. The reactions were monitored by TLC on silica-gel plates (Merck 60F254). Starting materials, solvents, and reagents were purchased from commercial sources and were used without further purification.

3.2. Synthesis of 2,6-diaryl-9,10-anthraquinones

*3.2.1. 2,6-Bistriflat of 2,6-dihydroxyanthracene-9,10-dione (**2**)*

In a dry round bottomed flask, under nitrogen, dry pyridine (20 mL) was added to 2,6-dihydroxyanthracene-9,10-dione (1 g, 4.163 mmol, 1.0 eq) while cooling the reaction mixture in ice bath, trifluoromethanesulfonic anhydride (1.55 mL, 9.242 mmol, 2.22 eq) was then added dropwise. The reaction mixture was stirred at RT for 15 h. The mixture was then poured into aqueous HCl (0.1 M, 100 mL) and extracted with dichloromethane (30 mL) three times. The organic phase was extracted with aqueous HCl (0.1 M, 50 mL) three times and water three times, dried using Na_2SO_4, filtered,

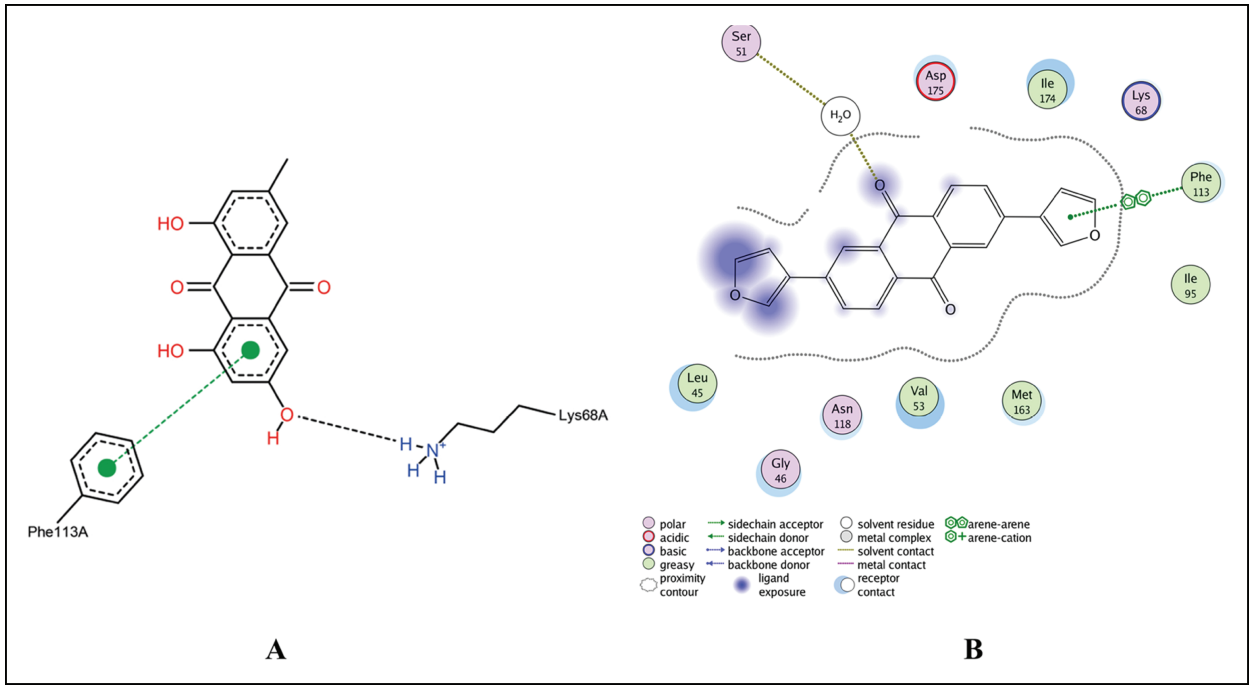


Fig. 5: A) 2D Interactions of emodin with the amino acid residues of the ATP-binding site of the human CK2α as shown in PDB. B) Docking of compound **3** with the active site pocket of human CKα.

then the solvent was evaporated under vacuum to yield a dark yellow solid. Recrystallization from toluene and isopropanol yield white gray needles (1.101 g, 52 %). The synthesis was performed according to Gautrot et al. (2007) with modification.

*3.2.2. 2,6-Di(furan-3-yl)anthracene-9,10-dione (**3**)*

The bistriflated compound (**2**) (292.5 mg, 0.58 mmol, 1.0 eq), 3-furanboronic acid (162.2 mg, 1.45 mmol, 2.5 eq) and tetrakis(triphenylphosphine)palladium(0) (67.02 mg, 0.058 mmol, 0.1 eq) were added in a 3-neck round bottomed flask and the reaction was performed under nitrogen. 20 mL THF and aqueous Na_2CO_3 (1 M, 5 mL) were added *via* septum. The mixture was heated under reflux for 18 h and then poured into aqueous HCl (0.1 M, 200 mL). The aqueous phase was extracted twice with dichloromethane, the extract was then washed with water three times and dried using Na_2SO_4. The solvent was evaporated under vacuum to yield a brown solid (185 mg). Flash column chromatography (cyclohexane/ethylacetate 4:1) was used to purify the mixture and gain compound **3** (60.9 mg, 0.18 mmol, 31 %). Preparative HPLC was used to purify the compound further using acetonitrile/H_2O/formic acid (80:20:0.1). The synthesis followed the procedure of (Gautrot et al. 2007) with modification, where a similar structure was developed but with furan-2-yl instead of furan-3-yl.

*3.2.3. 2,6-Di(pyrimidine-5-yl)anthracene-9,10-dione (**4**)*

The bistriflated compound (**2**) (300 mg, 0.59 mmol, 1.0 eq), 5-pyrimidineboronic acid (184.3 mg, 1.49 mmol, 2.5 eq) and tetrakis(triphenylphosphine)palladium(0) (68.7 mg, 0.06 mmol, 0.1 eq) were added in a 3-neck round bottomed flask and the reaction was performed under nitrogen. 20 mL THF and aqueous Na_2CO_3 (1 M, 5 mL) were added *via* septum. The mixture was heated under reflux for 18 h, then poured into aqueous HCl (0.1 M, 200 mL). The aqueous phase was extracted twice with dichloromethane, the extract was then washed with water three times and dried using Na_2SO_4. The solvent was evaporated under vacuum to yield a brown solid (190 mg). Flash column chromatography was used to purify the mixture (cyclohexane/ethylacetate 4:1, ethylacetate + 5 % methanol) (3.0 mg, 0.01 mmol, 2 %). The synthesis followed the procedure of (Gautrot et al. 2007) with modification.

*3.2.4. 2,6-Bistriflate of 2,6-dihydroxyanthracene-9,10-dione (**2**)*

^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm] = 8.48 (d, J = 8.6 Hz, 2H), 8.21 (d, J = 2.5 Hz, 2H), 7.74 (dd, J = 8.6, 2.6 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$): δ [ppm] = 180.0, 153.8, 135.5, 132.8, 130.8, 127.5, 120.4, 117.2. APCI-MS: *m/z* calculated $[C_{16}H_7F_6O_8S_2]^+$: 504,9481; found: 504.9530. M.P. 219 °C (Lit.: 219-221 °C) (Gautrot et al. 2007).

*3.2.5. 2,6-Di(furan-3-yl)anthracene-9,10-dione (**3**)*

^{1}H NMR (600 MHz, DMSO-d_6): δ [ppm] = 8.56 (s, 2H, H1/H14), 8.38 (d, J = 1.6 Hz, 2H, H4/H11), 8.23 (d, J = 8.0 Hz, 2H, H5/H12), 8.17 (dd, J = 8.1, 1.7 Hz, 2H, H18/H26), 7.86 (t, J = 1.7 Hz, 2H, H20/H24), 7.20 (s, 2H, H21/H23). ^{13}C NMR (151 MHz, DMSO-d_6): δ [ppm] = 181.9, 145.2, 141.9, 138.2, 133.9, 131.5, 130.9, 127.8, 124.6, 123.0, 108.6. APCI-MS: *m/z* calculated $[C_{22}H_{13}O_4]^+$: 341.0808; found: 341.0818. IR: $\tilde{\nu}$ [cm^{-1}] = 3129, 1667, 1593, 1555, 1516, 1477, 1358, 1327, 1312, 1288, 1250, 1219, 1173, 1157, 1115, 1088, 1057, 1022, 980, 922, 872, 849, 814, 741, 714, 671. M.P.: 276-278 °C.

3.3. Biological assay

The preparation of the human recombinant CK2 holoenzyme was performed according to a protocol previously described (Gratz et al. 2010). For the expression of the α-subunit (CSNK2A1) and β-subunit (CSNK2B) of the human protein kinase CK2 the pT7-7 expression system in *Escherichia coli* BL21 (DE3) was used. Fractions exhibiting CK2 activity were combined and analyzed by SDS-PAGE and Western Blot. The capillary electrophoresis based assay was used for testing the inhibitors of the human CK2 as described earlier (Gratz et al. 2010). 2 µL of the dissolved inhibitors (stock solution in DMSO) were mixed with 78 µL of CK2 supplemented kinase buffer which was composed of 1 µg CK2 holoenzyme, 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT. The reaction was initiated by the addition of 120 µL assay buffer, which was composed of 25 mM Tris/HCl (pH 8.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 100 µM ATP and 190 µM of the substrate peptide RRRDDDSDDD. The reaction was carried out for 15 min at 37 °C and stopped by the addition of 4 µL EDTA (0.5 M). Subsequently the reaction mixture was analyzed by a PA800 capillary electrophoresis from Beckman Coulter (Krefeld, Germany). Acetic acid (2 M, adjusted with conc. HCl to a pH of 2.0) was used as the electrolyte for electrophoretic separation. The separated substrate and product peptide were detected at 214 nm using a DAD-detector. IC_{50} values were calculated from the resulting dose–response curves. For the determination of the mode of inhibition, the ATP concentration in the assay buffer was varied to 10, 50 and 250 µM, while the rest of the procedure was identical to the IC_{50} determination described above.

*3.4. Molecular docking of compound 3 and human CK2*α

The crystal structure of human CK2alpha in complex with emodin (PDB code: 3C13), was used for performing the docking study applying the Protonate3D function in MOE. Subsequently the original ligand of the structure was omitted and the docking simulation was performed. For this purpose the receptor was defined by the receptor and solvent and the docking site was defined by the original ligand atoms. The ligand to be docked, compound **3**, was provided in a conformational database created by the Conformations

Import function in MOE. Triangle Matcher was chosen as the placement method and Rescoring 1 was set to London dG. Refinement was achieved by Forcefield and Rescoring 2 was set to London dG. All other parameters were kept at their default values.

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