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A new family of densely functionalized fused-benzoquinones as potent human protein kinase CK2 inhibitors

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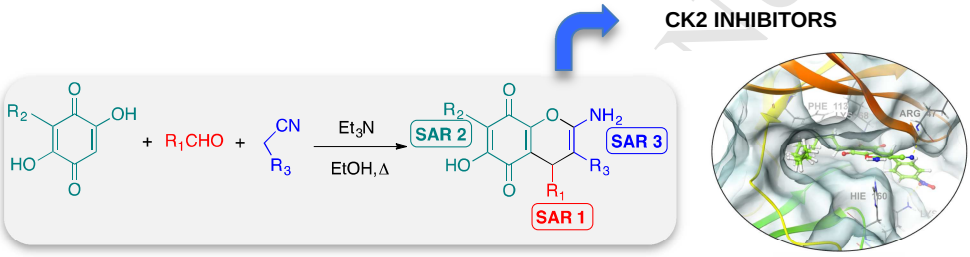
Abstract

A new series of 2-amino-4-phenyl-6-hydroxy-7-alkyl-pyranobenzoquinones was synthesized as ATP-competitive CK2 inhibitors. They were readily synthesized through a three-component Knoevenagel condensation-Michael addition-heterocyclization reaction from aldehydes, malononitrile, and 3-alkyl-2,5-dihydroxybenzoquinones. Some of the synthesized compounds presented interesting inhibitory activity with IC_{50} values in the submicromolar range. A structure-activity relationship study was carried out and the mode of binding was analysed by docking studies and supported by ATP competition assays.



1. Introduction

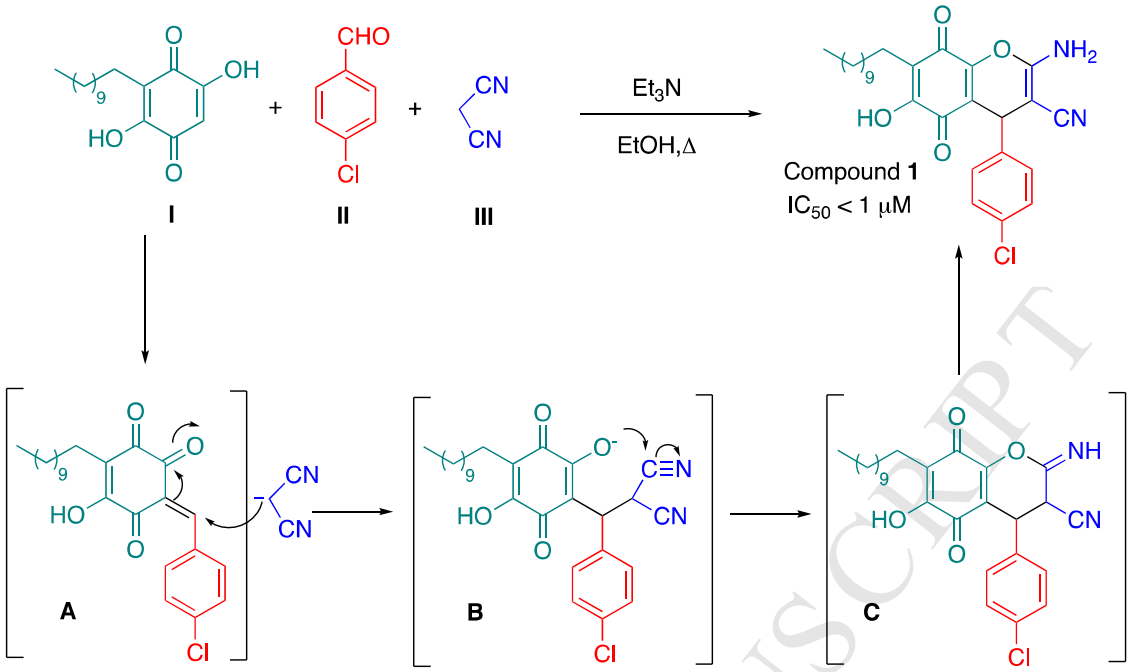
Protein kinase CK2 is a highly conserved serine/threonine protein kinase that is ubiquitously distributed in multiple cell types [1]. It is a holoenzyme comprises a heterotetramer consisting of two catalytic (α and/or α') and two regulatory (β) subunits. It is recognized as one of the most pleiotropic protein kinase with more than 300 substrates [2], many of which have been confirmed to play crucial roles in the dysfunction of programmed cell apoptosis and death, migration, differentiation and oncogenic transformation [3-6]. Overexpression of CK2 has been observed in many cancers, including hematologic cancers such as acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), T-cell acute lymphoblastic leukemia (T-ALL) and multiple myeloma (MM) [7-10]. Downregulation of CK2, either by transfection of specific siRNA or plasmid-based expression of kinase-inactive CK2, resulted in reduction of cancer cell viability and induction of apoptosis [11-13]. Thus, CK2 is becoming an important target for the treatment of cancer and other life-threatening diseases. Several compounds, belonging to different classes of chemical compounds like anthraquinones, xanthenones, coumarins, pyrazolo-triazynes, peptides, benzimidazoles and polyoxygenated benzotriazoles have been reported as CK2 inhibitors [14-23]. However until now, only one of them, the pyridoquinoline CX-4945, has entered in Phase II clinical trials as potencial anticancer drug [24-27].

Herein, we report the structural optimization and docking studies of a new family of ATP-competitive CK2 inhibitors with the 2-amino-3-cyano-4*H*-pyranobenzoquinone core.

2. Results and discussion

Most of the CK2 inhibitors known today are small and flat scaffolds having several rings, which fits into the active site of the CK2 and competes with ATP [14]. One of the representative CK_2 inhibitors is the anthraquinone emodin [28]. Due to our interest in antitumoral quinones [29-33], a set of 60 structurally diverse natural and synthetic quinones [31] was screened on CK2 activity. From this screening compound **1** emerged with an $IC_{50} < 1$ μM.

Compound **1** was obtained through a multicomponent reaction from the natural benzoquinone embelin (**I**) [34], 4-chlorobenzaldehyde (**II**) and malonotrile (**III**). Based on calculated values of the Fukui function (f^-) as local density functional descriptor [32], the synthesis of this compound can be rationalized by the initial formation of a conjugated electron-deficient enone (**A**) through a Knoevenagel condensation of embeline (**I**) and 4-chlorobenzaldehyde (**III**). The next step of this mechanism could involve a Michael addition of malononitrile (**II**) to the reactive quinone methide intermediate to yield the intermediate (**B**), which experiments an intramolecular cyclization to yield the intermediate C that tautomerizes to compound **1** (Scheme 1).



Scheme 1. Structure and Plausible Formation of Compound **1**.

Compound **1** was tested for its inhibitory activity towards the human CK2 holoenzyme following the procedure described earlier [22, 35]. The synthetic peptide RRRDDDSDDD was used as a substrate. For initial testing, inhibition was determined at inhibitor concentrations of 10 μM in DMSO as solvent. IC_{50} value was determined by measuring CK2 inhibition at eight different concentrations ranging from 0.001 to 100 μM in appropriate intervals and calculated from the resulting dose-response curve [35]. Compound **1** was active toward the target enzyme with an IC_{50} value of 0.90 ± 0.05 μM. As a control the well-known CK2 inhibitor, emodin was subjected to the same test conditions which had an IC_{50} value of 0.58 ± 0.05 μM (Fig. 1).

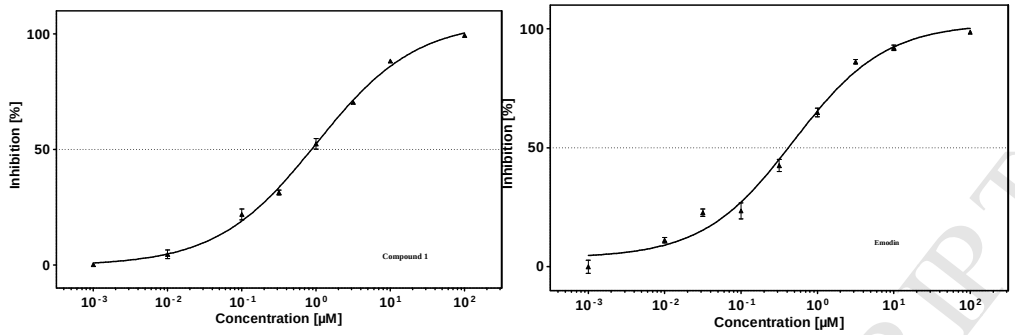


Fig. 1. IC_{50} value determination of compound **1** and emodin with human protein kinase CK2.

The binding pattern of **1** was analyzed by flexible molecular docking. The compound inserted into the narrow ATP binding site of CK2 (Fig. 2). As shown the aliphatic chain of **1** was located at the edge of the pocket and established van der Waals interactions with the side chains residues of Ile95, Met163, Val116, Val66 and Ile174. Two hydrogen bonds were observed between the hydroxyl group of **1** and Lys 68, and between the cyano group and Arg47. Other representative interaction was a π-π interaction between the aromatic ring and His160. Based on the binding mode, the presence of different substituents at the phenyl ring to enhance the contact with CK2, modifications in the length side chain to locate the ligand more deeply within the ATP binding site, and the replacement of the cyano moiety by other electron withdrawing groups may increase the inhibitory activity.

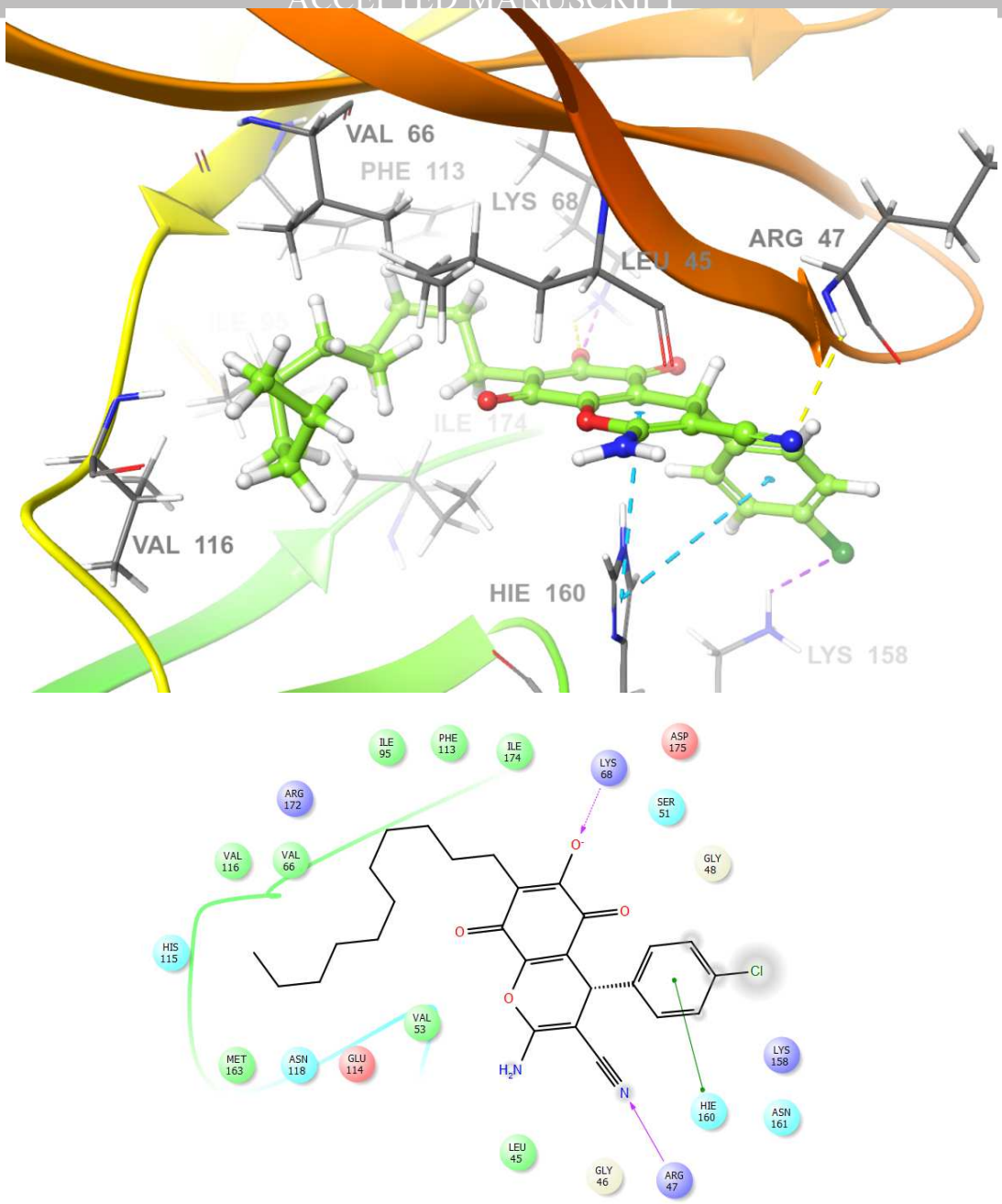
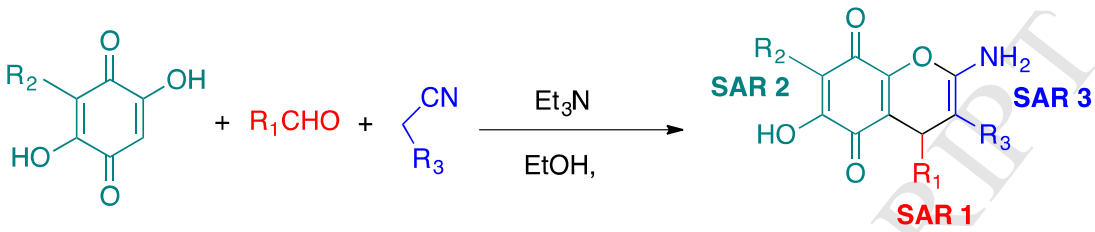


Fig. 2. Binding mode prediction of **1** with CK2 (PDB 3PE1).

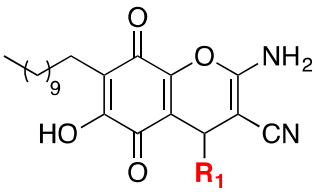
Thus, the SAR of this class of compounds was investigated by synthesizing structural variations at each of the three sites designated as SAR 1, SAR 2 and SAR 3.



Scheme 2. Modular modifications to SAR1, SAR2 and SAR3 compounds.

In the SAR 1 study, the synthesis was driven by preserving the original functionalities for the SAR 2 and SAR 3 regions in the lead compound, while the SAR 1 position was varied. Various functional groups, such as substituted phenyls, heterocycles and alkyls were introduced in SAR 1. Table 1 shows the structures of SAR 1 compounds and CK2 inhibitory activity.

Table 1. Structure of SAR 1 compounds and CK2 inhibitory activity



Compound	R_1	Inhibition (%)[a]	IC_{50} ± SD (μM)[b]
1	-Ph-4-Cl	81	0.90 ± 0.052
2	-Ph-4-Br	48	nd[c]
3	-Ph-4-F	50	nd
4	-Ph-3-F	74	0.996 ± 0.06
5	-Ph-3-F-4-OMe	49	nd
6	-Ph-4-CN	60	nd
7	-Ph-4-NO_2	90	0.22 ± 0.04

8	-Ph-3-NO_2	81	0.89 ± 0.024
9	-Ph-4-CF_3	58	nd
10	-Ph-4-COOH	88	1.36 ± 0.19
11	-Ph-4-COOMe	64	nd
12	-Ph	76	0.57 ± 0.038
13	-Ph-4-OMe	78	0.84 ± 0.16
14	-Ph-3,4-$(OMe)_2$	38	nd
15	-Ph-3,4-OCH_2O-	73	1.94 ± 0.16
16	-3-pyridin	71	1.38 ± 0.094
17	-4-pyridin	86	1.44 ± 0.4
18	-2-furyl	66	0.72 ± 0.016
19	-cyclohexyl	37	nd
20	-$C(CH_3)_3$	11	nd
21	-CH_2CH_3	ni[d]	nd
22	-$(CH_2)_5CH_3$	75	3.71 ± 0.079
Emodin		99	0.58 ± 0.05

[a] The percent inhibition of CK2 activity was determined for each compound at a fixed concentration of 10 μM.
[b] For the best compounds producing at least 65% inhibition at 10 μM, the concentration was varied to determine the IC_{50}.
[c] nd: not determined
[d] ni: no inhibition

The replacement of chlorine in *para* position by other halogens such as bromine or fluorine (Table 1, compounds **2**-**3**) led to loss of activity, while the activity was retained when the fluorine was located at *meta*-position. The effect of withdrawing groups like -CN, -NO_2, -CF_3, -COOH and -COOMe (Table 1, compounds **6**-**11**) was analysed. The introduction of a nitro group at the *para* position of the phenyl group (**7**) showed 4-fold improvement of the CK2 inhibitory activity compared to the lead compound **1**. Compound **8** having the nitro group at the *meta* position was as active as compound **1**, and derivative -Ph-4-COOH (**10**) resulted slightly less active than **1**. The rest of the compounds with electron-withdrawing groups (**6**, **9**, **11**) did not show good activity, with inhibition percentage lower than 65%. Compound **12** with an unsubstituted phenyl group showed a slightly better activity than compound **1**.

Regarding the analogues with electron-donating groups (**13-15**), compound **13** (-Ph-4-OMe) showed slightly better activity than **1**, and a loss of activity was observed with additional electron-donating groups (**14-15**). Substitutions of the phenyl groups with heterocyclic rings were also evaluated (compounds **16-18**), the best activity for this series was achieved with the 2-furyl derivative (0.72 ± 0.016) while 3-pyridin and 2-pyridin analogues resulted 1.4 and 1.6-fold less active than **1**. The introduction of non-aromatic moieties in the SAR1 position (**19-22**) resulted in loss of CK2 inhibitory activity.

On the basis of the SAR1 findings, a phenyl group or heteroaromatic ring was necessary for the activity, which is supported by the existence of a π-π interaction between the aromatic moiety and His-160 in the Docking model. The different substituents at *para*- or *meta*- position modulate the CK2 inhibitory activity depending on the effectiveness of the interactions they establish with the corresponding residues. Thus for instance, the best activity achieved by compound **7** having a 4-NO_2 group can be explained on the basis of four hydrogen bond interactions: between the amino group and Leu 45, between the cyano group and Arg 47, between the OH and Lys 68, and between the NO_2 group and Lys 158. The aliphatic chain of compound **7** was also located at the edge of the pocket in the same way as compound **1** and established van der Waals interactions with amino acid residues such as Ile95, Ile174, Val 116, Val66 and Phe113 (Fig. 3). Finally, the π-π interaction between the aromatic ring and His 160 was also observed. The interactions with His-160 and Lys-68 are similar to those observed for the potent CK2 inhibitor CX-4945.

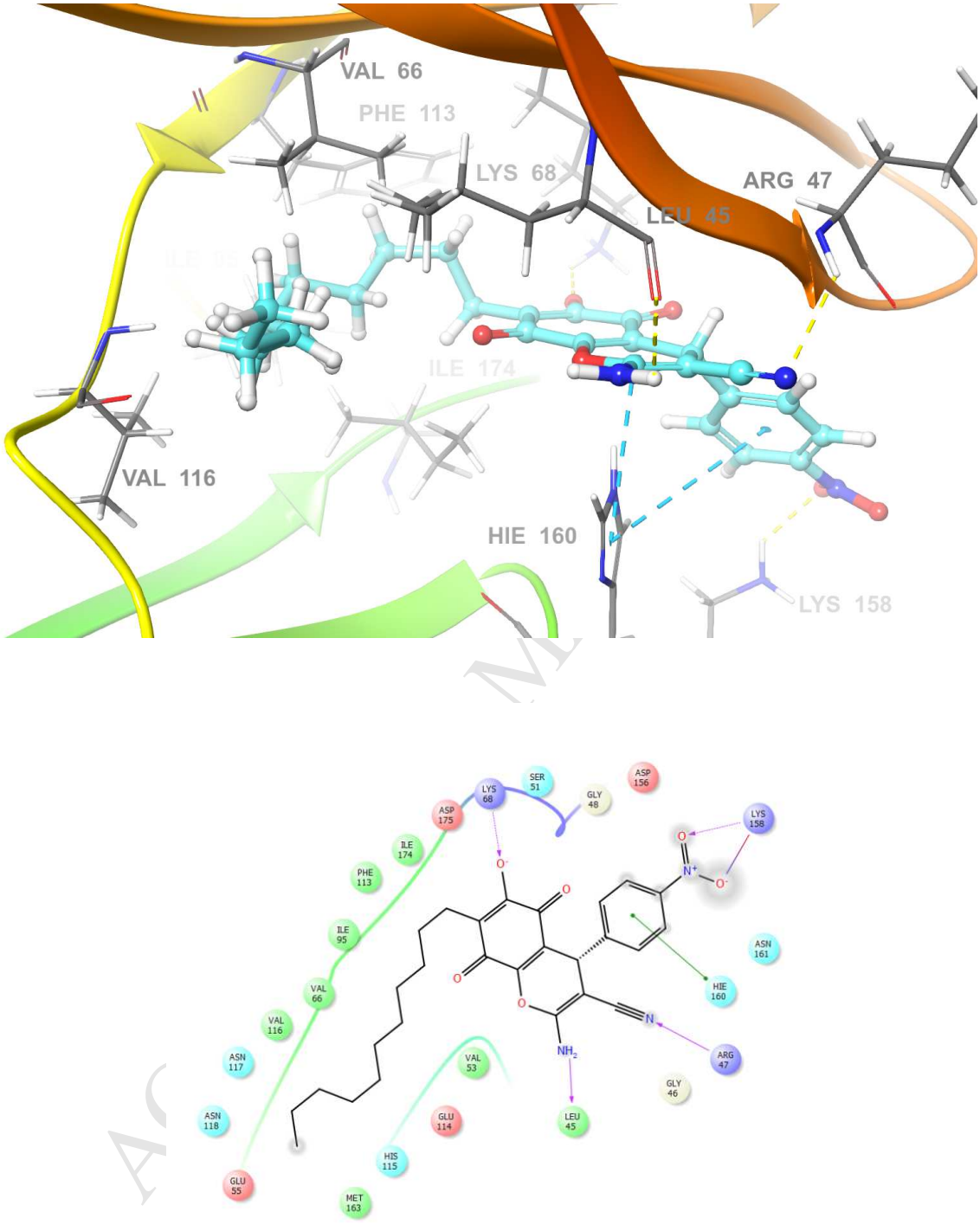
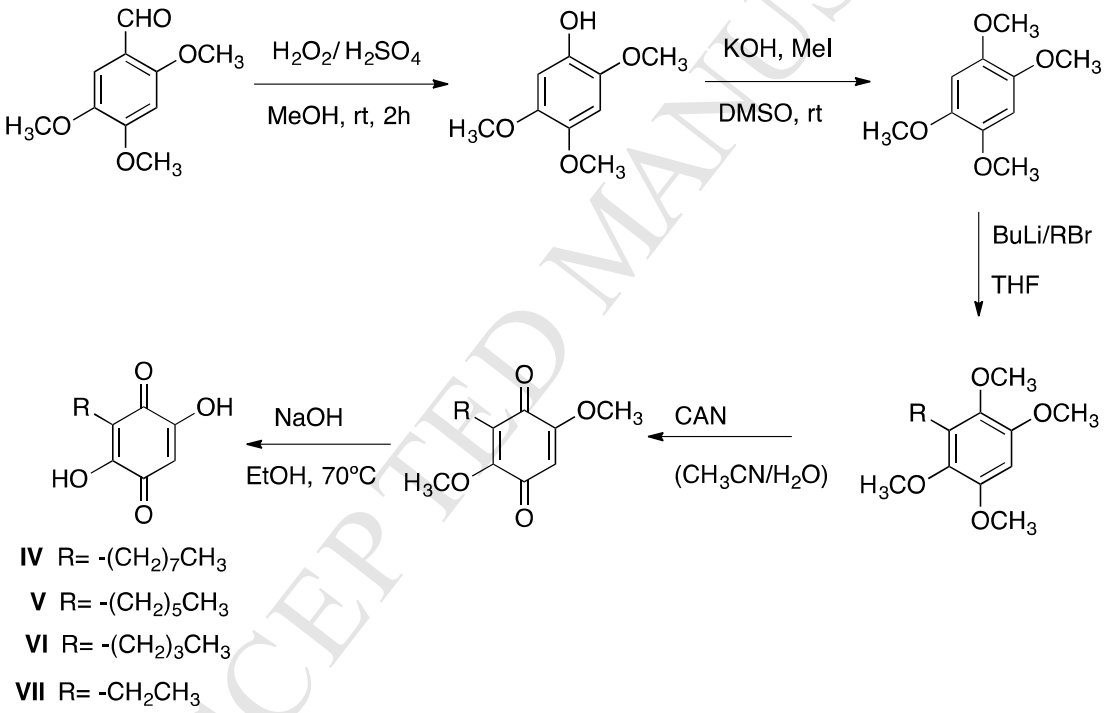


Fig. 3. Binding mode prediction of **7** with CK2 (PDB 3PE1).

Next, our exploration shifted to modifications at the lateral chain of the benzoquinone nucleus (SAR 2). Considering that the CK2 inhibitory activity is

favoured by the presence of the -Ph-4-NO_2 (SAR 1), the subsequent analogues were investigated with this moiety retained. The influence of the side chain length on the CK2 inhibitory was analysed with the preparation of compounds **23** (R_2= $(CH_2)_7CH_3$), **24** (R_2= $(CH_2)_5CH_3$), **25** (R_2= $(CH_2)_3CH_3$) and **26** (R_2= CH_2CH_3).

These compounds were obtained from the multicomponent reaction of 4-nitrobenzaldehyde, malononitrile and the corresponding benzoquinones **IV**, **V**, **VI** and **VII**. The benzoquinones (**IV-VII**) were synthesized following the reactions shown in Scheme 3 [36].

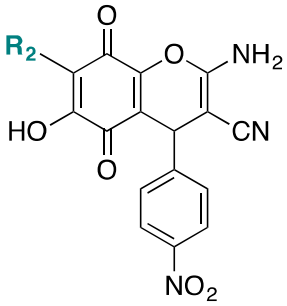


Scheme 3. Preparation of benzoquinones (**IV-VII**).

Table 2 shows the results obtained in the evaluation of derivatives (**23-26**). As we can see the shortening of the side chain produces a drastic loss of activity. In docking model, compound **24** (R_2=$(CH_2)_5CH_3$), for example, showed the same pose and similar

interactions as compound **7** into the binding pocket except for the interactions established by the alkyl chain. The inactivity of this compound could be explained for the absence of the hydrophobic interactions that the C-7-C-11 fragment of the alkyl chain of compound **7** establish, and reinforces the key role that the C-11 plays driving the orientation of the compounds within the lining of the ATP-binding site.

Table 2. Structure of SAR 2 compounds and CK2 inhibitory activity.



Compound	R_2	Inhibition (%)[a]	IC_{50} ± SD (μM)
6	$-(CH_2)_{10}CH_3$	90	0.22 ± 0.04
23	$-(CH_2)_7CH_3$	80	1.99 ± 0.19
24	$-(CH_2)_5CH_3$	ni[a]	nd[b]
25	$-(CH_2)_3CH_3$	26	nd
26	$-CH_2CH_3$	26	nd

[a] ni: no inhibition
[b] nd: not determined

Attending to the SAR 2 region we also evaluated the derivatives **27** and **28** in order to see the effect of the replacement of the alkyl chain and the hydroxyl group by a fused aromatic ring, and also the replacement of the free hydroxyl by a methoxy group. In both cases low percentages of inhibition were obtained (37% (**27**), 39% (**28**)), which ratifies the importance of the C-11 alkyl chain and the free hydroxyl group for the activity (Fig. 4).

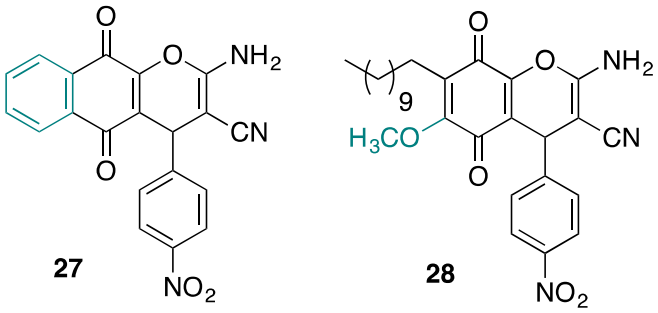


Fig. 4. Structures of derivatives **27** and **28**.

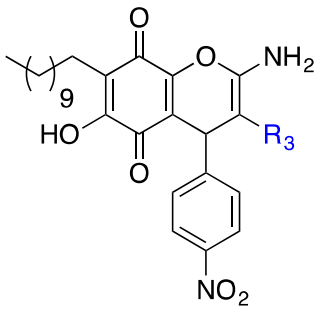
Next, our attention turned to the SAR 3 series bearing the -Ph-4-NO_2 (SAR 1), -$(CH_2)_{10}CH_3$ and the free hydroxyl (SAR 2) moieties on the structural template.

Since the NH_2 group in the docking model establishes a hydrogen bond interaction with Leu 45, the importance of this group for the activity was assumed, and the SAR 3 was focused in the replacement of the CN group with electron-withdrawing groups, which could also act as hydrogen bond acceptors.

Because of the CN group is introduced by the malononitrile component in the MCR, we carried out the MCR with other methylene active compound bearing one cyano group (necessary to generate the NH_2 functionality) and other electron-withdrawing groups such as methylformyloxy or ethylformyloxy. Replacement of the cyano moiety by these groups (**29**-**30**) resulted in loss of activity. Compounds **29** and **30** with a methylformyloxy and an ethylformyloxy groups, respectively, showed a similar pose respect to compound **7**. The carbonyl of the methylformyloxy and ethylformyloxy moieties presents the same interaction that the cyano group establishes with Arg 47. The loss of activity of these compounds could be explained considering a plausible intramolecular hydrogen bond between the NH_2 group and the corresponding methoxy

or ethoxy group, which consequently debilities the interaction between the amino group and Leu 45.

Table 3. Structure of SAR 3 compounds and CK2 inhibitory activity.



Compound	R_3	Inhibition (%)[a]	IC_{50} ± SD (μM)
7	-CN	90	0.22 ± 0.04
29	$-COOCH_3$	58	nd[a]
30	$-COOCH_2CH_3$	53	nd

[a] nd: not determine

The effect of the lead compound **1** (IC_{50}: 0.90 μM) on viability of MCF-7 breast cancer cells was tested using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazoliumbromide) assay and compared to the effect of compounds **4** (IC_{50}: 1 μM), 7 (0.22 μM), **8** (0.89 μM), **12** (0.57 μM), and **13** (0.84 μM). As it is shown in Fig. 5, all compounds tested showed almost no cytotoxic effect in MCF-7 cells after 48 h incubation at concentration of 10 μM. While using 100 μM (which is far away from any therapeutic dosage) the cytotoxicity was between 50-90%. The highest effect was shown by compound **7** with around 90 % inhibition, while compound **12** showed the weakest effect with around 50 %, and the inhibition effects of other compounds were for **4**, **8**, and **13** around 60%, 65%, and 55%, respectively.

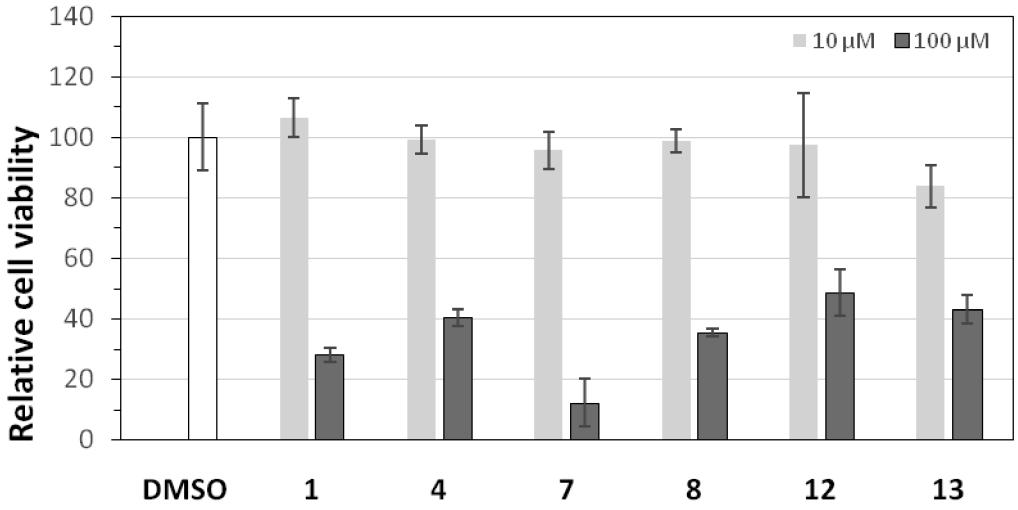


Fig. 5. Effects of five selected inhibitors (**1**, **4**, **7, 8**, **12**, and **13**) on the cell viability of MCF-7 cells evaluated by MTT assay, after 48h of incubation. Control cells contained 1% DMSO and the results are presented as mean values (± SD) of three assays. Columns in dark gray represents % cell viability after incubation with 100 µM of the compounds. Columns in light gray represents the effect in 10 µM.

In order to validate the ATP competitive mode of inhibition, compound **12** was selected as a representative inhibitor of this series, and the IC_{50} values were determined at eight different concentrations of compound **12** ranging from 0.001 to 100 µM, and repeated four times using different ATP concentrations each time. The IC_{50} values were observed to increase linearly with the ATP concentration, indicating the ATP competitive mode of CK2 inhibition (Fig. 6). The K_i value of CK2 inhibitory by compound **12** was determined from the IC_{50} values obtained at various ATP concentrations. The regression line showed a Y-axis intercept at an IC_{50} value of 0.39 µM (Fig. 6) and thereby defined the K_i value of compound **12** to be at this concentration.

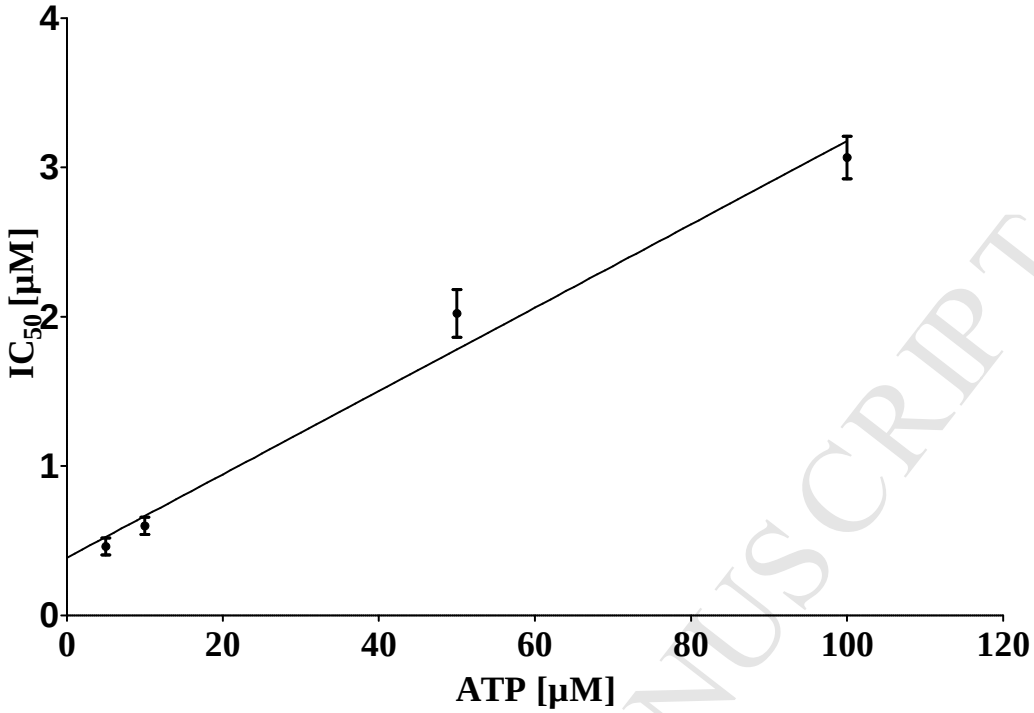


Fig. 6. ATP-competitive inhibition of human CK2 by compound **12**. Four IC_{50} values with different ATP concentrations were determined using eight different concentration of the inhibitor ranging from 0.001 to 100 µM and plotted against the respective ATP concentrations. Each IC_{50} value was determined 3 times independently. Mean values with corresponding standard deviation are given. The K_i value is defined as the Y-intercept and was calculated to be 0.39 µM (R^2= 0.93).

3. Conclusion

From the screening on CK_2 activity of a set of 60 structurally diverse natural and synthetic quinones, an embelin derivative (**1**) with an IC_{50} = 0.90 ± 0.052 µM was identified. The SAR of the compound class was investigated by synthesizing structural variations and by determination of their CK2 inhibitory activities. It was possible to enhance the activity of compound (**1**) and an IC_{50} value of 0.22 ± 0.04 µM was achieved for the most active compound. The cell viability for some compounds was determined, and the binding pattern of these compounds into the ATP binding site of CK2 was

analysed by molecular docking, and it also was corroborated by ATP competitive assay. This family of inhibitors is a promising start point for further chemical modifications focused on the nature of the heterocyclic ring fused to the benzoquinone core to obtain more efficient CK2 inhibitors.

4. Experimental Section

4.1 General Experimental Procedures. IR spectra were obtained using a Fourier Transform Infrared spectrometer. NMR spectra were recorded in $CDCl_3$ or DMSO at 500 or 600 MHz for ^{1}H NMR and 125 or 150 MHz for ^{13}C NMR. Chemical shifts are given in (δ) parts per million and coupling constants (*J*) in hertz (Hz). ^{1}H and ^{13}C spectra were referenced using the solvent signal as internal standard. Melting points were taken on a capillary melting point apparatus and are uncorrected. HREIMS were recorded using a high-resolution magnetic trisector (EBE) mass analyzer. Analytical thin-layer chromatography plates used were Polygram-Sil G/UV254. Preparative thin-layer chromatography was carried out with Analtech silica gel GF plates (20 x 20 cm, 1000 Microns) using appropriate mixtures of ethyl acetate and hexanes. All solvents and reagents were purified by standard techniques reported [37] or used as supplied from commercial sources. All compounds were named using the ACD40 Name-Pro program, which is based on IUPAC rules. The embelin (**1**) used in the reactions was obtained from *Oxalis erythrorhiza Gillies ex Hook. & Arn.* following the procedure described in reference [34].

4.2 General Procedures for the preparation of dihydropyran embeline derivatives

To a mixture of 0.1 mmol of malononitrile, 0.1 mmol of the corresponding aldehyde, and 10 mol % of Et_3N in 5 mL of EtOH were added 30 mg of embelin (0.1 mmol). The reaction mixture was stirred and refluxed until disappearance of the starting

benzoquinone. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC to yield the corresponding dihydropyran derivative.

4.2.1 2-Amino-4-(4-chlorophenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (1).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 14.3 mg of 4-chlorobenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 4.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 21.1 mg (54%) of **1** as an amorphous brown solid. Mp 154.7-155.4 °C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.25 (bs, 16H), 1.43 (m, 2H), 2.42 (t, *J*=7.3 Hz, 2H), 4.58 (s, 1H), 4.94 (bs, 2H, NH_2), 7.23 (d, *J*=7.9 Hz, 2H), 7.32 (d, *J*=7.9 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 3), 31.9 (CH_2), 35.6 (CH), 61.7 (C), 116.4 (C), 117.6 (C), 120.0 (C), 129.2 (CH x 2), 129.3 (CH x 2), 134.1 (C), 139.7 (C), 147.1 (C), 151.0 (C), 157.7 (C), 179.1 (C), 181.3 (C); EIMS *m/z* 482 ([M^+], 4), 418 (100), 294 (50), 188 (30), 154 (67); HREIMS 482.1967 (calcd. for $C_{27}H_{31}N_2O_4{}^{35}Cl$ [M^+] 482.1972); IR 3360, 3185, 2925, 2853, 2290, 2199, 2080, 1642, 1605, 1491, 1460, 1412, 1330, 1229, 1174, 1118, 1093, 1039, 1013, 838 cm^{-1}.

4.2.2 2-Amino-4-(4-bromophenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (2).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 18.9 mg of 4-bromobenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 2 h. The solvent was removed under vacuum, and the crude product was purified by

preparative-TLC with 30% Hex/EtOAc to yield 28.4 mg (54%) of **2** as an amorphous brown solid. Mp 185.5-186.6 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.3 Hz, 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.42 (t, *J*=7.6 Hz, 2H), 4.56 (s, 1H), 4.93 (bs, 2H, NH_2), 7.17 (d, *J*=8.5 Hz, 2H), 7.47 (d, *J*=8.2 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.6 (CH), 61.5 (C), 116.3 (C), 118.1 (C) 119.9 (C), 122.2 (C), 129.7 (CH x 2), 132.1 (CH x 2), 140.2 (C), 147.1 (C), 150.9 (C), 157.7 (C), 179.1 (C), 181.3 (C); EIMS *m/z* 427 ([M^+], 10), 462 (100), 371 (75), 294 (71), 153 (55), HREIMS: 526.1475 (calcd. for $C_{27}H_{31}N_2O_4{}^{79}Br$ [M^+] 526.1467); IR 3423, 3365, 3187, 2922, 2851, 2388, 2294, 2198, 2114, 1986, 1912, 1642, 1605, 1536, 1488, 1462, 1411, 1330, 1228, 1118, 1075, 1006, 837 cm^{-1}.

4.2.3 2-Amino-4-(4-fluorophenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (3).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 14.3 mg of 4-fluorobenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL. The reaction mixture was refluxed for 20 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 31.1 mg (65%) of **3** as an amorphous brown solid. Mp 168.4-169.2 °C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.42 (t, *J*=7.6 Hz, 2H), 4.59 (s, 1H), 4.92 (bs, 2H, NH_2), 7.07 (t, *J*=8.2 Hz, 2H), 7.26 (bs, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.4 (CH), 61.90 (C), 115.9 (CH x 2, *J*=21.5 Hz), 116.6 (C), 117.6 (C), 119.9 (C), 129.7 (CH x 2, *J*=8.1 Hz), 137.0 (C), 147.0 (C), 151.0 (C), 157.7 (C), 162.4 (C-F, *J*=240.45 Hz), 179.1 (C), 181.4 (C); EIMS *m/z* 466 ([M^+], 35), 401 (36),

371 (100), 232 (78), 149 (28); HREIMS 466.2268 (calcd. for $C_{27}H_{31}N_2O_4F$ [M^+] 466.2268); IR 3369, 3189, 2925, 2854, 2386, 2289, 2205, 2115, 1642, 1606, 1509, 1412, 1382, 1332, 1229, 1177, 1118, 1042, 1009, 842 cm^{-1}.

4.2.4 2-Amino-4-(3-fluorophenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (4).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 10.7 µL of 3-fluorobenzaldehide (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 21.5 mg (46%) of **4** as an amorphous brown solid. Mp 167.5-168.2°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.43 (t, *J*=7.6 Hz, 2H), 4.60 (s, 1H), 4.93 (bs, 2H, NH_2), 6.98 (m, 2H), 7.00 (bs, 1H), 7.10 (d, *J*= 7.53 Hz, 1H), 7.32 (m, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.5 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.8 (CH), 61.6 (C), 114.9 (CH), 115.1 (CH), 115.3 (C), 116.3 (C), 117.8 (C, *J*= 98 Hz), 119.9 (C), 123.6 (CH, *J*=3.0 Hz), 130.6 (CH, *J*= 7.9 Hz), 143.6 (C, *J*=7.6 Hz), 147.2 (C), 151.0 (C), 157.8 (C), 179.0 (C), 181.2 (C); ESMS(-) *m/z* 465 ([M^+-1], 100), 437 (7), 399 (7), 281 (10); HREIMS 465.2187 (calcd. for $C_{27}H_{30}N_2O_4F$ [M^+-1] 465.2190); IR 2924, 2854, 2203, 1639, 1593, 1493, 1450, 1377, 1234, 1122, 1045, 972, 894, 717, 675, 648 cm^{-1}.

4.2.5 2-Amino-4-(3-fluoro-4-methoxyphenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (5).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.7 mg of 3-fluor-4-methoxybenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was

refluxed for 20 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 19 mg (38%) of **5** as an amorphous brown solid. Mp 157.5-158.0 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.24 (bs, 16H), 1.43 (m, 2H), 2.42 (t, *J*=7.9 Hz, 2H), 3.86 (s, 3H), 4.53 (s, 1H), 4.95 (bs, 2H, NH_2), 6.93 (t, *J*=8.5 Hz, 1H), 6.99 (dd, *J*=11.6, 1.8 Hz, 1H), 7.04 (d, *J*=8.5 Hz, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.2 (CH), 56.3 (CH_3), 61.6 (C), 113.5 (CH), 115.6 (CH, *J*=19.3 Hz), 116.4 (C), 117.7 (C), 119.9 (C), 123.9 (CH, *J*=3.1 Hz), 134.1 (C, *J*=5.0 Hz), 146.9 (C), 147.5 (C, *J*=10.2 Hz), 151.0 (C), 152.4 (C-F, *J*=248.1 Hz), 157.7 (C), 179.2 (C), 181.4 (C); EIMS *m/z* 496 ([M^+], 20), 432 (37), 371 (28), 232 (43), 154 (47), 66 (100); HRMS 496.2385 (calcd. for $C_{28}H_{33}N_2O_5F$ [M^+] 496.2374); IR 3789, 3387, 3190, 2924, 2853, 2390, 2292, 2204, 1881, 1681, 1640, 1515, 1461, 1438, 1411, 1382, 1329, 1273, 1227, 1120, 1032, 977, 823 cm^{-1}.

4.2.6 2-Amino-4-(4-cyanophenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (6).

Following the general procedure, 35 mg of embelin (0.12 mmol) was added to a mixture of 15.6 mg of 4-formylbenzonitrile (0.12 mmol), 7.9 mg of malononitrile (0.12 mmol) and 2 µL of Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 50.5 mg (89%) of **6** as an amorphous brown solid. Mp 176.9-178.1°C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.87 (t, *J*=7.1 Hz, 3H), 1.24 (bs, 16H), 1.43 (m, 2H), 2.43 (t, *J*=7.8 Hz, 2H), 4.66 (s, 1H), 4.98 (bs, 2H, NH_2), 6.92 (bs, 1H), 7.42 (d, *J*=8.0 Hz, 2H), 7.66 (d, *J*=8.0 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 29.6

(CH_2 x 4), 29.6 (CH_2), 31.9 (CH_2), 36.2 (CH), 60.8 (C), 112.1 (C), 115.7 (C), 117.3 (C), 118.3 (C), 120.3 (C), 128.8 (CH x 2), 132.9 (CH x 2), 146.2 (C), 147.4 (C), 151.0 (C), 157.9 (C), 178.8 (C), 181.1 (C); EIMS *m/z* 473 ([M^+], 4), 409 (100), 373 (3), 268 (41), 153 (12); HREIMS 473.2342 (calcd. for $C_{28}H_{31}N_3O_4$ [M^+] 473.2315); IR 3399, 2924, 2854, 2233, 2203, 1643, 1620, 1516, 1373, 1308, 1054, 987, 972, 852, 818 cm^{-1}.

4.2.7 2-Amino-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (7).

Following the general procedure described above, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.4 mg of 4-nitrobenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10% mol) in 5 mL of EtOH. The reaction mixture was refluxed for 1.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 47 mg (94%) of **7** as an amorphous brown solid. Mp 147.9-148.3 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*= 6.8 Hz, 3H), 1.24 (bs, 16H), 1.43 (m, 2H), 2.43 (t, *J*= 7.5 Hz, 2H), 4.71 (s, 1H), 5.07 (bs, 2H, NH_2), 6.94 (bs, 1H, OH), 7.48 (d, *J*=8.5 Hz, 2H), 8.21 (d, *J*=8.5 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2 x 2), 29.5 (CH_2 x 2), 29.6 (CH_2 x 2), 31.9 (CH_2), 36.0 (CH), 60.6 (C), 115.6 (C), 117.4 (C), 120.3 (C), 124.3 (CH x 2), 129.0 (CH x 2), 147.5 (C), 147.6 (C), 148.0 (C), 151.0 (C), 158.0 (C), 178.8 (C), 181.2 (C); EIMS *m/z* (%) 430 ([M^+-NO_2,-OH], 31), 429 (100), 289 (44), 154 (30); ESHRMS(-) 492.2143 (calcd. for $C_{27}H_{30}N_3O_6$ [M-H]$^+$ 492.2135; IR 3393, 3196, 2927, 2856, 2293, 2206, 2119, 1934, 1646, 1609, 1522, 1348, 1235, 1117, 1048, 979, 863, 834 cm^{-1}.

4.2.8 2-Amino-6-hydroxy-4-(3-nitrophenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (8).

Following the general procedure, 33 mg of embelin (0.11 mmol) was added to a mixture of 16.8 mg of 3-nitrobenzaldehyde (0.11 mmol), 7.4 mg of malononitrile (0.11 mmol) and 2 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 50.5 mg (91%) of **8** as an amorphous brown solid. Mp 213.1-214.8°C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.88 (t, *J*=7.1 Hz, 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.44 (t, *J*=7.7 Hz, 2H), 4.74 (s, 1H), 5.03 (bs, 2H, NH_2), 6.92 (bs, 1H, OH), 7.55 (t, *J*=7.8 Hz, 1H), 7.69 (d, *J*=7.6 Hz, 1H), 8.13 (t, *J*= 1.8 Hz, 1H), 8.16 (dd, *J*= 1.9, 8.2Hz, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 36.0 (CH), 60.4 (C), 115.6 (C), 117.4 (C), 120.3 (C), 123.0 (CH), 123.2 (CH), 129.9 (CH), 134.4 (CH), 143.3 (C), 147.4 (C), 148.7 (C), 151.0 (C), 158.0 (C), 178.8 (C), 181.2 (C); EIMS *m/z* 493 ([M^+], 28), 429 (100), 371 (43), 294 (37), 153 (41); HREIMS 493.2198 (calcd. for $C_{27}H_{31}N_3O_6$ [M^+] 493.2213); IR 3387, 3337, 2924, 2854, 1643, 1609, 1524, 1504, 1381, 1346, 1288, 1057, 987, 972, 822, 667 cm^{-1}.

4.2.9 2-Amino-6-hydroxy-5,8-dioxo-4-(4-(trifluoromethyl)phenyl)-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (9).

Following the general procedure, 33 mg of embelin (0.1 mmol) was added to a mixture of 15.3 µL of 4-(trifluoromethyl)benzaldehyde (0.1 mmol), 7.4 mg of malononitrile (0.1 mmol) and 2 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 47.1 mg (81%) of **9** as an amorphous brown solid. Mp 164.8-166.0°C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.88 (t, *J*=7.4 Hz, 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.43 (t, *J*=7.7 Hz, 2H), 4.67 (s, 1H), 4.98 (bs, 2H, NH_2), 6.95 (bs, 1H, OH), 7.42 (d, *J*=7.8 Hz, 2H), 7.62 (d, *J*=8.2 Hz, 2H); ^{13}C-

NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.9 (CH), 61.2 (C), 116.1 (C), 117.5 (C), 120.1 (C), 123.8 (C, *J*= 275.4 Hz), 126.0 (CH x 2, *J*=3.4 Hz), 128.4 (CH x 2), 130.3 (C, *J*= 32.7Hz), 145.0 (C), 147.3 (C), 151.0 (C), 157.8 (C), 178.9 (C), 181.2 (C); ESHRMS(-) 515.2157 (calcd. for $C_{28}H_{30}N_2O_4F_3$ [M^+-1] 515.2158); IR 3398, 2924, 2854, 1670, 1620 1500, 1408, 1377, 1323, 1064, 972, 875, 806, 737, 667 cm^{-1}.

4.2.10 4-(2-Amino-3-cyano-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromen-4-yl)benzoic acid (10).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.3 mg of 4-formylbenzoic acid (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 40.1 mg (80%) of **10** as an amorphous brown solid. Mp 198.1-199.8°C; H-NMR (500 MHz, DMSO-d_6) δ 0.86 (t, *J*=6.7 Hz, 3H), 1.23 (bs, 16H), 1.32 (m, 2H), 2.26 (t, *J*=7.7 Hz, 2H), 4.51 (s, 1H), 7.28 (bs, 2H, NH_2), 7.38 (d, *J*=7.9 Hz, 2H), 7.89 (d, *J*= 7.9 Hz, 2H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ 13.9 (CH_3), 22.1 (CH_2 x 2), 28.4 (CH_2), 28.7 (CH_2), 29.0 (CH_2), 29.1 (CH_2 x 2), 29.2 (CH_2), 29.3 (CH_2), 31.3 (CH_2), 35.9 (CH), 57.0 (C), 78.9 (C), 114.6 (C), 119.5 (C), 127.6 (CH x 2), 128.1 (C), 129.2 (C), 129.6 (CH x 2), 146.6 (C), 149.0 (C), 158.9 (C), 167.1 (C); ESMS(-) *m/z* 491 ([M^+-1], 100), 425 (50), 413 (28), 281 (35), 245 (60); ESHRMS 491.2181 (calcd. for $C_{28}H_{31}N_2O_6$ (M^+-1) 491.2182); IR 3518, 3398, 1682, 1666, 1620 1493, 1412, 1381, 1053, 978, 798, 667 cm^{-1}.

4.2.11 Methyl 4-(2-amino-3-cyano-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromen-4-yl)benzoate (11).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 14.8 mg of methyl 4-formylbenzoate (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 2 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 38.6 mg (75%), of **11** as an amorphous brown solid. Mp 146.6-147.9°C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.24 (bs, 16H), 1.43 (m, 2H), 2.42 (t, *J*=7.9 Hz, 2H), 3.90 (s, 3H), 4.65 (s, 1H), 4.69 (bs, 2H, NH_2), 6.94 (bs, 1H, OH), 7.37 (d, *J*=8.1 Hz, 2H), 8.01 (d, *J*=8.3 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 36.0 (CH), 52.1 (CH_3), 61.3 (C), 116.2 (C), 117.5 (C), 119.1 (C), 128.0 (CH x 2), 129.9 (C), 130.3 (CH x 2), 146.0 (C), 147.3 (C), 151.0 (C), 157.8 (C), 166.5 (C), 179.0 (C), 181.2 (C); ESHRMS(-) 505.2336 (calcd. for $C_{29}H_{33}N_2O_6$ [M^+-1] 505.2339); IR 3399, 2924, 2854, 2198, 1682, 1616, 1447, 1380, 1284, 1064, 968, 667 cm^{-1}.

4.2.12 2-Amino-6-hydroxy-5,8-dioxo-4-phenyl-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (12).

Following the general procedure described above, 30 mg of embelin (0.1 mmol) was added to a mixture of 10.8 mg of benzaldehyde (10.4 µL, 0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 13.6 mg (30%) of **12** as an amorphous brown solid. Mp 155.9-156.6 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.3 Hz, 3H), 1.24 (bs, 16H), 1.43 (m, 2H), 2.42 (t, *J*=7.6 Hz, 2H), 4.59 (s, 1H), 4.91 (bs, 2H, NH_2), 7.27 (m, 3H), 7.34 (m, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5

(CH_2), 29.6 (CH_2 x3), 31.9 (CH_2), 36.0 (CH), 62.1 (C), 116.8 (C), 119.8 (C), 127.9 (CH x 2), 128.1 (CH), 129.0 (CH x 2), 141.2 (C), 147.1 (C), 151.0 (C), 157.7 (C), 179.3 (C), 181.4 (C); EIMS *m/z* 448 ([M^+], 20), 384 (39), 371 (100), 214 (32); HREIMS 448.2357 (calcd. for $C_{27}H_{32}N_2O_4$ [M^+] 448.2362); IR 3373, 2918, 2851, 2203, 1680, 1641, 1607, 1496, 1454, 1382, 1330, 1226, 1175, 1117, 998, 835 cm^{-1}.

4.2.13 2-Amino-6-hydroxy-4-(4-methoxyphenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (13).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 13.9 mg of 4-methoxybenzaldehyde (12.5 µL, 0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 9 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 22.9 mg (47%) of **13** as an amorphous brown solid. Mp 154.1-154.6 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.88 (t, *J*=7.0 Hz, 3H), 1.25 (bs, 16H), 1.43 (m, 2H), 2.42 (t, *J*=7.9 Hz, 2H), 3.78 (s, 3H), 4.54 (s, 1H), 4.85 (bs, 2H, NH_2), 6.86 (d, *J*=8.5 Hz, 2H), 7.20 (d, *J*=8.5 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.5 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.2 (CH), 55.3 (CH_3), 66.4 (C), 114.4 (CH x 2), 116.9 (C), 117.9 (C), 119.7 (C), 129.1 (CH x 2), 133.4 (C), 146.8 (C), 150.9 (C), 157.5 (C), 159.4 (C), 179.3 (C), 181.5 (C); EIMS *m/z* (%) 478 ([M^+], 0.3), 414 (100), 274 (20), 147 (19); HREIMS 478.2475 (calcd. for $C_{28}H_{34}N_2O_5$ [M^+] 478.2468); IR 3356, 3188, 2923, 2853, 2388, 2290, 2202, 2079, 1897, 1681, 1641, 1608, 1511, 1462, 1411, 1382, 1331, 1230, 1176, 1118, 1037, 836 cm^{-1}.

4.2.14 2-amino-4-(3,4-dimethoxyphenyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (14).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 17 mg of 3,4-dimethoxybenzaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 4 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 33.5 mg (65%) of **14** as an amorphous brown solid. Mp 150.2-151.9°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.43 (t, *J*=7.8 Hz, 2H), 3.85 (s, 3H), 3.89 (s, 3H), 4.54 (s, 1H), 4.90 (bs, 2H, NH_2); 6.81 (m, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.6 (CH), 55.9 (CH_3), 56.0 (CH_3), 62.2 (C), 111.3 (CH), 111.4 (CH), 116.8 (C), 117.9 (C), 119.1 (C), 119.7 (C), 120.1 (CH), 133.8 (C), 146.8 (C), 148.9 (C), 149.2 (C), 151.0 (C), 157.6 (C), 179.4 (C), 181.5 (C); EIMS *m/z* 508 ([M^+], 43), 444 (31), 414 (33), 294 (68), 153 (100); HREIMS 508.2558 (calcd. for $C_{29}H_{36}N_2O_6$ [M^+] 508.2573); IR 3360, 3797, 3005, 2924, 2877, 2368, 2198, 2168, 1978, 1732, 1643, 1516, 1462, 1327, 1254, 1231, 1126, 1026, 883, 818, 675 cm^{-1}.

4.2.15 2-Amino-4-(benzo[d][1,3]dioxol-5-yl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (15).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.3 mg of piperonal (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 9 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 5% DCM/EtOAc to yield 10.7 mg (22%) of **15** as an amorphous brown solid. Mp 126.5-127.2 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz 3H), 1.25 (bs, 16H), 1.44 (m, 2H), 2.42 (t, *J*=7.6 Hz, 2H), 4.51 (s, 1H), 4.91 (bs, 2H, NH_2), 5.95 (d, *J*=0.9 Hz, 2H), 6.75 (m, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2),

22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 35.7 (CH), 62.2 (C), 101.3 (CH_2), 108.3 (CH), 108.5 (C), 108.6 (CH), 116.7 (C), 119.8 (C), 121.5 (CH), 135.2 (C), 146.9 (C), 147.4 (C), 148.2 (C), 151.0 (C), 157.6 (C), 179.2 (C), 181.4 (C); EIMS *m/z* 492.5 ([M^+], 3), 216 (100), 172 (11), 114 (23), 69 (14); HRMS: 492.2274 (calcd. for $C_{28}H_{32}N_2O_6$ [M^+] 492.2260); IR 3363, 3188, 2923, 2854, 2205, 1681, 1646, 1605, 1503, 1447, 1412, 1389, 1333, 1232, 1118, 1041, 1001, 928, 864 cm^{-1}.

4.2.16 2-Amino-6-hydroxy-5,8-dioxo-4-(pyridin-3-yl)-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (16).

Following the general procedure, 33 mg of embelin (0.1 mmol) was added to a mixture of 10.5 µL of 3-pyridinecarboxaldehyde (0.1 mmol) and 7.4 mg of malononitrile (0.1 mmol) in 5 mL of ethanol and 2 µL de Et_3N (10% mol). The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 37.1 mg (74%) of **16** as an amorphous brown solid. Mp 159.7-161.0°C; ^{1}H-NMR (500 MHz $CDCl_3$) δ 0.86 (t, *J*=7.1 Hz, 3H), 1.24 (bs, 16H), 1.44 (m, 2H), 2.44 (t, *J*=7.6 Hz, 2H), 4.79 (s, 1H), 5.19 (bs, 2H, NH_2), 7.35 (dd, *J*= 4.9, 7.9 Hz, 1H), 7.67 (dt, *J*=1.9, 7.8 Hz, 1H), 8.44 (d, *J*=4.3 Hz, 1H), 9.02 (s, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2 x 2), 31.9 (CH_2), 33.8 (CH), 59.6 (C), 116.0 (C), 117.8 (C), 120.4 (C), 124.3 (CH), 136.6 (CH), 137.5 (C), 146.6 (CH), 147.8 (C), 149.7 (CH), 153.9 (C), 158.9 (C), 179.2 (C), 181.6 (C); ESMS *m/z* 449 ([M^+], 30), 448 (100), 329 (8), 255 (10), 212 (22); HRMS 448.2227 (calcd. for $C_{26}H_{30}N_3O_4$ [M^+] 448.2236); IR 3425, 3402, 3174, 2924, 2854, 2203, 1674, 1635, 1597, 1500, 1327, 1231, 1115, 1045, 806, 756, 709 cm^{-1}.

4.2.17 2-Amino-6-hydroxy-5,8-dioxo-4-(pyridin-4-yl)-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (17).

Following the general procedure, 35 mg of embelin (0.12 mmol) was added to a mixture of 11.3 µL of 4-pyridinecarboxaldedhyde (0.12 mmol), 7.9 mg of malononitrile (0.1 mmol) and 2 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 40.2 mg (74%) of **17** as an amorphous brown solid. Mp 155.6-157.1°C; ^{1}H-NMR (500 MHz CD_3OD) δ 0.89 (t, *J*=7.4 Hz, 3H), 1.28 (bs, 16H), 1.44 (m, 2H), 2.41 (t, *J*=7.7 Hz, 2H), 4.61 (s, 1H), 7.39 (d, *J*= 5.9 Hz, 1H), 8.48 (d, *J*=5.5 Hz, 1H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 13.0 (CH_3), 21.9 (CH_2), 22.3 (CH_2), 27.7 (CH_2), 29.1 (CH_2 x 2), 29.2 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 31.7 (CH_2), 35.9 (CH), 56.4 (C), 115.0 (C), 118.3 (C), 119.5 (C), 123.4 (CH x 2), 147.9 (C), 148.9 (CH x 2), 153.1 (C), 154.3 (C), 159.4 (C), 179.1 (C), 181.3 (C); ESHRMS(-) 448.2232 (calcd. for $C_{26}H_{30}N_3O_4$ [M^+] 448.2236); IR 3379, 3317, 3209, 2924, 1854, 2198, 1686, 1643, 1596, 1415, 1331, 1234, 1211, 1119, 114, 914, 837, 686, 633 cm^{-1}.

4.2.18 2-Amino-4-(furan-3-yl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (18).

Following the general πrocedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 9.8 mg of 3-furaldehyde (8.5 µL, 0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 9 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 16.2 mg (36%) of **18** as an amorphous brown solid. Mp 142.6-143.5 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=7.0 Hz, 3H), 1.25 (bs, 16H), 1.45 (m, 2H), 2.44 (t, *J*=7.9 Hz, 2H), 4.75 (s, 1H), 4.91 (bs, 2H, NH_2),

6.29 (m, 1H), 6.33 (m, 1H), 7.32 (d, *J*=0.9 Hz, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH), 59.5 (C), 107.6 (CH), 110.8 (CH), 114.2 (C), 119.9 (C), 142.6 (CH), 147.8 (C), 150.9 (C), 152.2 (C), 15678 (C), 158.4 (C), 179.0 (C), 181.2 (C); EIMS *m/z* 438 ([M^+], 10), 374 (24), 232 (22), 66 (57), 57 (100); HREIMS 438.2166 (calcd. for $C_{25}H_{30}N_2O_5$ [M^+] 438.2155); IR 3749, 3614, 3383, 3193, 2922, 2852, 2393, 2213, 1681, 1644, 1379, 1331, 1235, 1203, 1145, 1121, 1073, 1013, 925, 886 cm^{-1}.

4.2.19 2-Amino-4-cyclohexyl-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (19).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 11.4 mg of cyclohexanaldehyde (12.4 µL, 0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 5.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 33.1 mg (71%) of **19** as an amorphous brown solid. Mp 156.2-157.1 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*=6.1 Hz, 3H), 0.97 (m, 1H), 1.25 (bs, 18H), 1.45 (m, 5H), 1.70 (m, 5H), 2.43 (t, *J*=6.4 Hz, 2H), 3.45 (s, 1H), 4.90 (bs, 2H, NH_2), 7.12 (bs, 1H), 6.99 (dd, *J*=11.6, 1.8 Hz, 1H), 7.04 (bs, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 26.0 (CH_2), 26.5 (CH_2), 27.8 (CH_2), 28.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 3), 29.7 (CH_2), 30.5 (CH_2), 31.9 (CH_2), 35.5 (CH), 44.1 (CH), 57.5 (C), 117.8 (C), 119.4 (C), 119.6 (C), 149.1 (C), 151.0 (C), 160.1 (C), 179.2 (C), 181.8 (C); EIMS *m/z* 454 ([M^+], 14); 388 (100); 371 (67); 359 (20); 231 (31); 167 (21); HREIMS 454.2816 (calcd. for $C_{27}H_{38}N_2O_4$ [M^+] 454.2832); IR 3329, 3209, 3182, 2922, 2852,

2390, 2292, 2194, 2084, 1677, 1634, 1600, 1518, 1451, 1405, 1336, 1295, 1237, 1116, 1061, 1002, 974, 948, 893 cm^{-1}.

4.2.20 2-Amino-4-(*tert*-butyl)-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (20). Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 11.1 µL of pivalaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 8 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 18 mg (41%) of **20** as an amorphous brown solid. Mp 162.1-163.0 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.88 (m, 12H), 1.25 (bs, 16H), 1.46 (m, 2H), 2.44 (t, *J*=7.9 Hz, 2H), 3.34 (s, 1H), 4.93 (bs, 2H, NH_2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 26.8 (CH_3 x 3), 28.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2 x2), 29.6 (CH_2 x 2), 31.9 (CH_2), 39.1 (CH), 39.6 (C), 58.7 (C), 118.6 (C), 119.4 (C), 119.4 (C), 150.1 (C), 151.0 (C), 161.1 (C), 179.2 (C), 182.2 (C); EIMS *m/z* 413 ($(M\text{-}CH_3)^+$, 3), 372 (72), 231 (100), 149 (13); HREIMS 413.2432 (calcd. for $C_{24}H_{32}N_2O_4$ [M^+-CH_3] 413.2440); IR 3333, 3205, 2921, 2852, 2194, 1638, 1597, 1519, 1465, 1407, 1332, 1239, 1191, 1116, 1033, 1016, 996, 886 cm^{-1}.

4.2.21 2-Amino-4-ethyl-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (21).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 8 µL of propanaldehyde (0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10% mol) in 5 mL of EtOH. The reaction mixture was refluxed for 2.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 16.6 mg (41%) of **21** as an amorphous brown solid.

Mp 164.2-165.1 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (m, 6H), 1.25 (bs, 16H), 1.45 (m, 2H), 1.73 (m, 2H), 2.44 (t, *J*=7.6 Hz, 2H), 3.61 (t, *J*=4.9 Hz, 1H), 4.81 (bs, 2H, NH_2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 9.0 (CH_3), 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 27.3 (CH_2), 28.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 30.9 (CH_2), 31.9 (CH), 59.6 (C), 117.0 (C), 118.3 (C), 119.7 (C), 148.9 (C), 151.0 (C), 159.0 (C), 179.1 (C), 181.8 (C); EIMS *m/z* 371 ([M^+-CH_3CH_2], 2), 334 (100), 196 (41), 165 (18); HREIMS 371.1979 (calcd. for $C_{21}H_{27}N_2O_4$ [M^+-CH_3CH_2] 371.1971); IR 3343, 3188, 2865, 2922, 2853, 2202, 1996, 1730, 1680, 1639, 1607, 1529, 1459, 1396, 1339, 1300, 1263, 1232, 1119, 1044, 994, 869 cm^{-1}.

4.2.22 2-Amino-4-heptyl-6-hydroxy-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (22).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 11.6 mg of heptanaldehyde (14.5 µL, 0.1 mmol), 6.7 mg of malononitrile (0.1 mmol) and 1.4 µL de Et_3N (10% mol), in 5 mL of EtOH. The reaction mixture was refluxed for 10.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 32.1 mg (41%) of **22** as an amorphous brown solid. Mp 152.0.-153.0 °C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (m, 6H), 1.25 (bs, 24H), 1.45 (m, 2H), 1.66 (m, 2H), 2.44 (t, *J*=7.6 Hz, 2H), 3.58 (t, *J*=4.3 Hz, 1H), 4.82 (bs, 2H, NH_2), 7.10 (bs, 1H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.0 (CH_3), 14.1 (CH_3), 22.6 (CH_2 x 2), 22.7 (CH_2), 24.9 (CH_2), 28.0 (CH_2), 29.1 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 30.0 (CH_2), 31.7 (CH_2), 31.9 (CH_2), 35.0 (CH), 59.9 (C), 117.6 (C), 119.6 (C), 148.6 (C), 151.0 (C), 159.9 (C), 179.2 (C), 181.8 (C); EIMS *m/z* 456 ([M^+], 16), 391 (54), 371 (100), 333 (71), 232 (23), 179 (19); HREIMS 456.2994 (calcd. for $C_{27}H_{40}N_2O_4$ [M^+] 456.2988); IR 3368, 3188, 2921, 2852,

2290, 2203, 2112, 1681, 1647, 1608, 1463, 1408, 1342, 1290, 1237, 1181, 1118, 1072, 1032, 1010, 972 cm^{-1}.

4.2.23 2-Amino-6-hydroxy-4-(4-nitrophenyl)-7-octyl-5,8-dioxo-5,8-dihydro-4H-chromene-3-carbonitrile (23).

Following the general procedure described above, 30 mg of 2,5-dihydroxy-3-octylcyclohexa-2,5-diene-1,4-dione (0.12 mmol) was added to a mixture of 18 mg of 4-nitrobenzaldehyde (0.12 mmol), 7.9 mg of malononitrile (0.12 mmol) and 2 µL of Et_3N (10% mol) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 33.8 mg (62%) of **23** as an amorphous brown solid. Mp 161.0-162.1°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*= 6.9 Hz, 3H), 1.25 (bs, 10H), 1.44 (m, 2H), 2.43 (t, *J*= 7.6 Hz, 2H), 4.72 (s, 1H), 5.03 (bs, 2H, NH_2), 6.92 (bs, 1H, OH), 7.48 (d, *J*=8.3 Hz, 2H), 8.21 (d, *J*=8.3 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.2 (CH_2), 29.3 (CH_2), 29.6 (CH_2), 31.8 (CH_2), 36.0 (CH), 60.7 (C), 115.6 (C), 117.3 (C), 120.3 (C), 124.3 (CH x 2), 129.0 (CH x 2), 147.4 (C), 147.6 (C), 148.0 (C), 151.0 (C), 157.9 (C), 178.8 (C); 181.2 (C); EIMS *m/z* (%) 451 ([M^+], 17), 387 (100), 329 (37), 289 (50), 153 (54); HRMS 451.1740 (calcd. for $C_{24}H_{25}N_3O_6$ [M^+] 451.1743); IR 3387, 3332, 3217, 2931, 2858, 2199, 1639, 1600, 1516, 1334, 1230 1168, 1111, 1045, 991, 879, 829, 717, 694 cm^{-1}.

4.2.24 2-Amino-7-hexyl-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-5,8-dihydro-4H-chromene-3-carbonitrile (24).

Following the general procedure described above, 30 mg of 3-hexyl-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione (0.13 mmol) was added to a mixture of 20.2 mg of 4-nitrobenzaldehyde (0.13 mmol), 8.8 mg of malononitrile (0.13 mmol) and 2 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1.5 h.

The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 41 mg (72%) of **24** as an amorphous brown solid. Mp 261.0-262.8°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*= 7.3 Hz, 3H), 1.28 (bs, 8H), 1.42 (m, 2H), 2.43 (t, *J*= 7.7 Hz, 2H), 4.72 (s, 1H), 5.03 (bs, 2H, NH_2), 6.94 (bs, 1H, OH), 7.48 (d, *J*= 7.9 Hz, 2H), 8.21 (d, *J*=7.9 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.5 (CH_2), 22.6 (CH_2), 28.0 (CH_2), 29.2 (CH_2), 31.5 (CH_2), 36.0 (CH), 60.7 (C), 115.6 (C), 117.3 (C), 120.3 (C), 124.3 (CH x 2), 129.0 (CH x 2), 147.4 (C), 147.6 (C), 148.0 (C), 151.0 (C), 157.9 (C), 178.8 (C), 181.2 (C); ESHRMS(-) 422.1349 (calcd. for $C_{22}H_{20}N_3O_6$ [M^+] 422.1352); IR 3514, 3398, 2958, 2928, 2858, 1682, 2635, 1620, 1519, 1458, 1346, 1053, 991, 968, 868, 825, 667 cm^{-1}.

4.2.25 2-Amino-7-butyl-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-5,8-dihydro-4H-chromene-3-carbonitrile (25).

Following the general procedure described above, 30 mg of 3-butyl-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione (0.15 mmol) was added to a mixture of 23.1 mg of 4-nitrobenzaldehyde (0.15 mmol), 10.1 mg of malononitrile (0.15 mmol) and 2.1 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 54.3 mg (90%) of **25** as an amorphous brown solid. Mp 242.6-244.0°C; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 0.86 (t, *J*= 8.7 Hz, 3H); 1.27 (m, 2H); 1.33 (m, 2H); 2.29 (t, *J*= 8.9 Hz, 2H); 4.65 (s, 1H); 7.58 (d, *J*= 8.6 Hz, 2H); 8.16 (d, *J*=8.8 Hz, 2H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ 14.2 (CH_3), 22.2 (CH_2), 22.6 (CH_2), 30.2 (CH_2), 36.4 (CH), 56.8 (C), 115.5 (C), 119.4 (C), 119.5 (C), 123.9 (C), 124.2 (CH x 2), 129.7 (CH x 2), 147.0 (C), 147.8 (C), 151.3 (C), 158.9 (C), 179.5 (C), 181.8 (C); ESHRMS (-) 394.1036 (calcd. for $C_{20}H_{16}N_3O_6$ [M^+] 394.1039). IR

3514, 3398, 3047, 2985, 2908, 1751, 1681, 1620 1558, 1519, 1450, 1384, 1057, 991, 972, 937, 875, 845, 821, 667 cm^{-1}.

4.2.26 2-Amino-7-ethyl-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-5,8-dihydro-4H-chromene-3-carbonitrile (26).

Following the general procedure described above, 30 mg of 3-ethyl-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione (0.18 mmol) was added to a mixture of 27 mg of 4-nitrobenzaldehyde (0.18 mmol), 11.8 mg of malononitrile (0.18 mmol) and 2.5 µL de Et_3N (10 mol %) in 5 mL of EtOH. The reaction mixture was refluxed for 1.5 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 30% Hex/EtOAc to yield 27.4 mg (42%) of **26** as an amorphous brown solid. Mp 194.2-196.0°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.08 (t, *J*= 7.4 Hz, 3H), 2.48 (q, *J*= 7.5 Hz, 2H), 4.73 (s, 1H), 4.99 (bs, 2H), 6.92 (bs, 1H), 7.49 (d, *J*= 8.6 Hz, 2H), 8.22 (d, *J*=8.6 Hz, 2H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 12.4 (CH_3), 29.7 (CH_2), 36.0 (CH), 60.8 (C), 115.7 (C), 124.3 (CH), 124.6 (CH), 128.6 (C), 129.0 (CH), 130.3 (C), 147.7 (C), 148.0 (C), 150.6 (C), 158.2 (C), 178.7 (C), 181.3 (C); EIMS *m/z* 367 ([M^+], 26), 303 (12), 245 (100), 153 (3), 66 (18); HREIMS 367.0827 (calcd. for $C_{18}H_{13}N_3O_6$ [M^+] 367.0804); IR 3394, 3348, 3325, 3251, 3190, 2974, 2198, 1766, 1643, 1608, 1519, 1415, 1353, 1923, 1311, 1238, 1068, 987, 914,875, 829, 694, 667, 652 cm^{-1}.

4.2.27 2-Amino-4-(4-nitrophenyl)-5,10-dioxo-5,10-dihydro-4H-benzo[g]chromene-3-carbonitrile (27).

To a solution of *p*-nitrobenzaldehyde (152 mg, 1.0 mmol), malononitrile (67 mg, 1.0 mmol) and 10 mol % of Et_3N in 5 mL of CH_3CN, 174 mg of 2-hydroxynaphthalene-1,4-dione (1.0 mmol) was added, and the reaction mixture was stirred for 24 h. After completion of the reaction, the precipitated product was separated by filtration and washed with *n*-hexane. The desired product was obtained as an orange powder (273 mg,

yield 73%) and showed identical spectroscopic data than those published in reference [38]. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 4.81 (s, 1H), 7.45 (s, 2H), 7.65 (d, *J*= 8.7 Hz, 2H), 7.84 (m, 3H), 8.06 (d, *J*=7.6 Hz, 1H), 8.16 (d, *J*= 8.7 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 36.4 (CH), 56.3 (C), 118.9 (C; 120.6 (C), 123.7 (CH x 2), 125.8 (CH), 126.1 (CH), 129.1 (CH x 2), 130.7 (C), 130.9 (C), 134.2 (CH), 134.5 (CH), 146.5 (C), 149.4 (C), 151.0 (C), 158.4 (C), 176.7 (C), 182.5 (C).

4.2.28 2-Amino-6-methoxy-4-(4-nitrophenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carbonitrile (28).

To 20 mg of **7** (0.04 mmol) dissolved in 3 mL of a mixture diethyl ether/MeOH (2:1) and excess of trimethylsilyldiazomethane (Me_3SiCHN_2) was added. The reaction mixture was stirred at room temperature until disappearance of starting material (48 h). The solvent was removed under reduce pressure and the product **28** was obtained in a quantitative yield without further purifications (20.3 mg, 100%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.88 (t, *J*= 6.9 Hz, 3H), 1.25 (bs, 16H), 1.37 (m, 2H), 2.42 (t, *J*= 8.2 Hz, 2H), 3.94 (s, 3H), 4.71 (s, 1H), 4.96 (bs, 2H, NH_2), 7.47 (d, *J*=8.7 Hz, 2H), 8.21 (d, *J*=8.8 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.7 (CH_2), 23.0 (CH_2), 28.6 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 36.1 (CH), 60.7 (C), 61.5 (CH_3), 117.4 (C), 117.5 (C), 124.3 (CH x 2), 128.9 (CH x 2), 131.0 (C), 146.2 (C), 147.5 (C), 148.5 (C); 155.3 (C); 158.0 (C); 179.3 (C); 181.0 (C); EIMS: *m/z* (%) 507 ([M^+], 60), 443 (94), 385 (100), 308 (43); HRMS 507.2385 (calcd. for $C_{28}H_{33}N_3O_6$ [M^+] 507.2369); IR 2417, 3333, 3240 ,2924, 2854, 2206, 1674, 1600, 1519, 1470, 1346, 1226, 1064, 968, 856, 806, 706, 667 cm^{-1}.

4.2.29 Methyl 2-amino-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carboxylate (29).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.4 mg of 4-nitrobenzaldehyde (0.1 mmol), 13.5 µL of methyl 2-cyanoacetate (0.1 mmol) and 2 µL of Et_3N (10% mol) in 5 mL of EtOH. The reaction mixture was refluxed for 22 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 22.2 mg (41%) of **29** as an amorphous brown solid. Mp 173.0-174.8°C; ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*= 7.2 Hz, 3H), 1.24 (bs, 16H), 1.42 (m, 2H), 2.41 (t, *J*= 7.6 Hz, 2H), 3.60 (s, 3H), 4.96 (s, 1H), 6.94 (bs, 1H), 7.48 (d, *J*=8.5 Hz, 2H), 8.13 (d, *J*=8.5 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 3), 31.9 (CH_2), 34.7 (CH), 51.4 (CH_3), 118.3 (C), 119.8 (C), 123.7 (CH x 2), 129.0 (C), 129.3 (CH x 2), 147.0 (C), 147.5 (C), 150.9 (C), 151.0 (C), 158.5 (C), 168.3 (C), 179.4 (C), 181.6 (C); EIMS *m/z* 526 ([M^+], 80), 467 (20), 429 (100), 404 (70), 294 (50), 153 (60); HREIMS 526.2340 (calcd. for $C_{28}H_{34}N_2O_8$ [M^+] 526.2315); IR 3468, 3382, 2924, 2851, 1697, 1620, 1512, 1439, 1346, 1227, 1072, 972, 918, 875, 845, 806, 706, 667 cm^{-1}.

4.2.30 Ethyl 2-amino-6-hydroxy-4-(4-nitrophenyl)-5,8-dioxo-7-undecyl-5,8-dihydro-4H-chromene-3-carboxylate (30).

Following the general procedure, 30 mg of embelin (0.1 mmol) was added to a mixture of 15.4 mg of 4-nitrobenzaldehyde (0.1 mmol), 16.3 µL of ethyl 2-cyanoacetate (0.1 mmol) and 2 µL de Et_3N (10% mol) in 5 mL of EtOH. The reaction mixture was refluxed for 19 h. The solvent was removed under vacuum, and the crude product was purified by preparative-TLC with 40% Hex/EtOAc to yield 24.9 mg (45%) of **30** as an amorphous brown solid. Mp 158.1-159.2°C. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.87 (t, *J*= 6.6 Hz, 3H), 1.12 (t, *J*= 7.1 Hz, 3H), 1.24 (bs, 16H), 1.42 (m, 2H), 2.41 (t, *J*= 7.5 Hz, 2H), 4.03 (q, *J*= 4.1 Hz, 2H), 4.96 (s, 1H), 6.95 (bs, 1H), 7.48 (d, *J*=8.7 Hz, 2H), 8.13

(d, *J*=8.5 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 14.1 (CH_3), 14.2 (CH_3), 22.6 (CH_2), 22.7 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2 x 2), 29.7 (CH_2), 31.9 (CH_2), 34.9 (CH), 60.2 (CH_2), 118.2 (C), 119.7 (C), 123.6 (CH x 2), 129.4 (C), 129.4 (CH x 2), 146.9 (C), 147.5 (C), 150.9 (C), 151.2 (C), 158.3 (C), 167.9 (C), 179.4 (C), 181.6 (C); ESHRMS(-) 539.2397 (calcd. for $C_{29}H_{35}N_2O_8$ [M^+-1] 539.2393); IR 3464, 3391, 292, 2854, 1693, 1628, 1516, 1427, 1346, 1281, 1192, 1065, 972, 875, 860, 802 cm^{-1}.

4.3 Biological evaluation

4.3.1. Inhibition of human CK2 holoenzyme

All compounds in this study were tested for their inhibitory activity towards the human CK2 holoenzyme following the procedure described earlier [22]. The synthetic peptide (RRRDDDSDDD) by CK2 was used as the substrate. The purity of the CK2 holoenzyme was more than 99 % which was prepared according to a protocol previously described [23]. 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 human CK2 as described earlier [23]. For initial testing, inhibition was determined relative to the controls at inhibitor concentrations of 10 μM in DMSO as a solvent. Therefore, 2 μL of the dissolved inhibitors were mixed with 78 μL of CK2 supplemented kinase buffer which was composed of 1 μg CK2 holoenzyme, 50 mMTris/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 contains 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. Pure solvent was used as negative control (0% inhibition), assays without the enzyme were used as positive control (100% inhibition). Compounds with at least 65% inhibition at 10 µM were used for IC_{50} determinations. IC_{50} values were calculated from the resulting dose-response curves [35], Prism 6 (GraphPad Software) was used to evaluate the IC_{50} values. For the determination of the mode of inhibition, the ATP concentration in the assay buffer was varied to 5, 10, 50 and 100 µM, while the rest of the procedure was identical to the IC_{50} determination described above.

4.3.2. Cell viability assay

The effect of CK2 inhibitors on the viability of MCF-7 cells was evaluated using MTT assay [39]. MCF-7 breast cancer cells (which were provided by the Department of Clinical Radiology of the University Hospital Münster, Germany), were cultured in RPMI 1640 medium containing GlutaMax (Life Technologies) and 10% fetal calf serum. MTT assay was performed in 96-well plates. Cells were seeded at a density of 1 x 10^5 cells per well and were incubated for 24 or 48 h at 37 °C in a humidified atmosphere (5% CO_2). After overnight incubation, seeding medium was removed and replaced with fresh medium containing the inhibitor at 10 or 100 µM. DMSO, at a final concentration of 1%, served as a control. Afterwards MTT reagent (Sigma Aldrich, Germany) was added at a final concentration of 0.5 mg/mL. After incubation for 2 h at 37 °C medium was discarded and 200 µL DMSO were added in order to solubilize the

formazan. After mixing, the absorption was determined using a microplate reader at 570 nm with a reference wavelength of 630 nm. The assay was performed in triplicates, and the experiments were repeated three times.

4.4. Protein Preparation and Docking.

The X-ray coordinates protein kinase CK2 alpha subunit were extracted from the Protein Data Bank (PDB code 3Q9X, 3R0T, 3OWJ, 5H8E, 3AT4, and 3PE1).

The PDB structures were prepared for docking using the Protein Preparation Workflow (Schrodinger, LLC, New York, NY, 2017) accessible from within the Maestro program (Maestro, version 11.1; Schrodinger, LLC: New York, NY, 2017). The substrate and water molecules were removed beyond 5 Å, bond corrections were applied to the cocrystallized ligands and an exhaustive sampling of the orientations of groups was performed. Finally, the receptors were optimized in Maestro 11.1 by using OPLS3 force field before docking study. In the final stage the optimization and minimization on the ligand-protein complexes were carried out with the OPLS3 force field and the default value for rmsd of 0.30 Å for non-hydrogen atoms were used. The receptor grids were generated using the prepared proteins, with the docking grids centered on the center of the bound ligand for each receptor. A receptor grid was generated using a 1.00 van der Waals (vdW) radius scaling factor and 0.25 partial charge cutoff. The binding sites were enclosed in a grid box of 20 $Å^3$ with default parameters and without constrains. The three-dimensional structures of the ligands to be docked were generated and prepared using LigPrep as implemented in Maestro 11.1 (LigPrep, version 2.9; Schrodinger, LLC: New York, NY, 2017) to generate the most probable ionization states at pH 7 ± 1 (retain original ionization state). These conformations were used as the initial input structures for the docking. In this stage a series of treatments are applied to the

structures. Finally the geometries are optimized using OPLS3 force field. These conformations were used as the initial input structures for the docking.

The ligands were docked using the extra precision mode (XP) [40] without using any constraints and a 0.80 van der Waals (vdW) radius scaling factor and 0.15 partial charge cutoff. The dockings were carried out with flexibility of the residues of the pocket near to the ligand. The generated ligand poses were evaluated with empirical scoring function, GlideScore a modified version of ChemScore [41], GlideScore implemented in Glide, was used to estimate binding affinity and rank ligands [42]. The XP Pose Rank was used to select the best- docked pose for each ligand. The best correlation with the CK2 inhibition and the best values of docking score was achieved when the 3PE1 was used.

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Highlights

- A new series of densely functionalized fused-benzoquinones was synthesized through a straight three-component reaction.
- Some compounds showed CK2 inhibition with IC_{50} values in the submicromolar range.
- Structure-activity relationships were outlined.
- The binding pattern was analysed by molecular docking, and corroborated by ATP competition assay.