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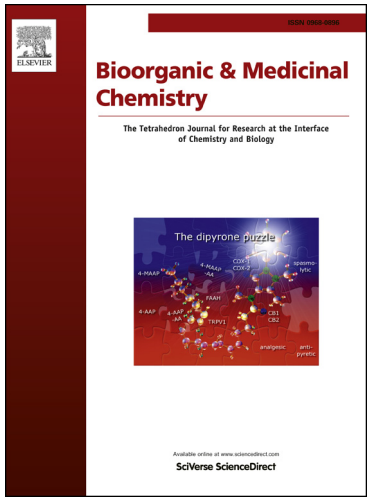

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Synthesis and biological activity of amino acid derivatives of avarone and its model compound

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ABSTRACT

A series of eighteen derivatives of marine sesquiterpene quinone avarone and its model system *tert*-butylquinone with amino acids has been synthesized by nucleophilic addition of amino acids to the quinones. *In vitro* cytotoxic activity toward human cancer cell lines (HeLa, A549, Fem-X, K562, MDA-MB-453) and normal MRC-5 cell line was determined. Several compounds showed very strong inhibitory activity with IC_{50} values less than 10 μM. Avarone derivatives were more active than the corresponding *tert*-butylquinone derivatives. The results of the cytofluorimetric analysis of cell cycle of HeLa cells showed that apoptosis might be one of possible mechanism of action of these compounds in cancer cells. In order to examine the influence of caspases on cell death, the apoptotic mechanisms induced by the tested compounds were determined using specific caspases 3, 8 and 9 inhibitors. For all compounds antibacterial activities against six strains of Gram-positive and four strains of Gram-negative bacteria were determined, as well as antifungal activity against three fungal species.

Keywords: Avarone, Amino acids, Cytotoxicity, Caspase, Apoptosis, Antimicrobial activity

1. Introduction

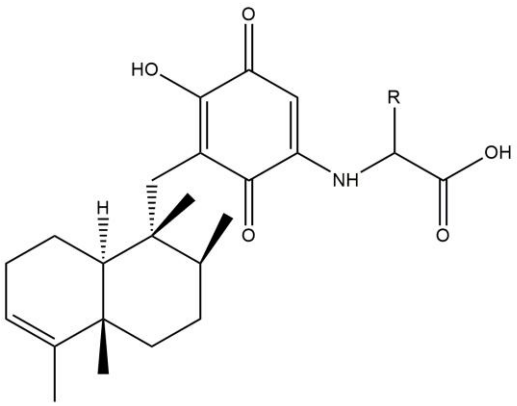
Marine organisms represent a rich source of metabolites with a high pharmacological potential and exceptionally different structures [**1-4**]. Many natural products with a decalin-type system and a quinoid moiety are important compounds with powerful and diverse biological properties [**5-7**].

Sponges of the order Dictyoceratida are a rich source of bioactive secondary metabolites, sesquiterpene quinones and hydroquinones such as avarol, avarone, illimaquinone, nakijiquinone and bolinaquinone. These compounds have attracted considerable interest due to their remarkable biological activities - antiproliferative, cytotoxic, antiviral and antimicrobial properties. All the above features of marine products provide the possibility for the further development of new agents which can be successfully identified as new drugs and/or factors which participate in clarifying intracellular events.

The hydroquinone avarol and its quinone derivative, avarone, were isolated from the marine sponge *Dysidea avara* [**8,9**]. This redox couple has a large range of pharmacological properties including antitumor [**10-12**], antiinflammatory [**13-15**], antibacterial [**16,17**], antiviral [**18,19**], antioxidant [**20,21**], antipsoriatic [**22,23**] and antibiofouling [**24**] activities. Also, the compounds induced DNA damage [**5,25-27**] as a result of reactive oxygen radicals generation, as well as enzyme inhibitory [**28-30**] activities incurred as a consequence of modification of biomolecules by nucleophilic addition.

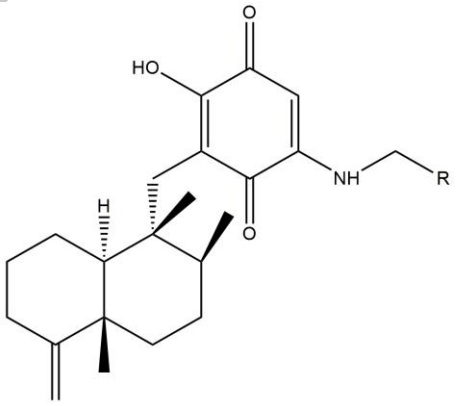
During the search for new metabolites from marine organisms, nakijiquinones have been isolated. Nakijiquinones have identical sesquiterpene skeleton as avarone, including the position of the double bond, and additional hydroxyl and either amino acid or amine substituents at the quinone moiety. Kobayashi et al. have isolated a great number of these bioactive metabolites from the Okinawan marine sponge extracts (family Spongiidae). Nakijiquinones A and B (which possess glycine and valine residue attached to the quinone ring, respectively, as shown in **Fig. 1**) were the first isolated sesquiterpenoid quinones with a normal amino acid residue from natural origin [**31**]. It should be emphasized that the presence of aminoquinone compounds is not very rare in natural sources [**32-34**]. Nakijiquinone A and B show a noticeable cytotoxicity against L 1210 murine leukemia cells and KB human epidermoid carcinoma cells, as well as antifungal

activity against fungi *Candida albicans* and *Aspergillus niger* [**31**]. Nakijiquinones C and D (**Fig. 1**), containing serine and threonine residue, respectively, were isolated from the same sponge family and display similar cytotoxicity [**35**]. Further research resulted in the isolation of two dimeric sesquiterpenoid quinones, nakijiquinones E and F, which did not show cytotoxicity against murine leukemia P388 and L1210, and KB human epidermoid carcinoma cell [**36**]. Unlike these, sesquiterpenoid quinones containing a different amine residue derived from amino acids, nakijiquinones G-I, showed a modest cytotoxicity against the forementioned cancer cells and an inhibitory activity against HER2 kinase [**37**]. Additionally, nakijiquinone H demonstrated a good antibacterial and antifungal activity [**37**]. Some of the nakijiquinones J-R, with amine residue attached to quinone ring, showed inhibitory activities against EGFR and HER2 tyrosine kinases [**38**]. Biologically active smenospongines (**Fig. 1**), isomeric to nakijiquinones, were isolated from the marine sponge *Dactylospongia elegans* [**39**] and showed a cytotoxic activity against a panel of tumor cell lines.



Nakijiquinone

A R = H **B** R = $CH(CH_3)_2$ **C** R = CH_2OH **D** R = $CH(OH)CH_3$



Smenospongine

B R = COOH **C** R = CH_2COOH

Fig. 1. Sesquiterpene quinones with an amino acid in side chain (nakijiquinones A–D; smenospongines B and C)

Considering all of the above, the rationale of this work was to obtain avarone derivatives with amino acids, which could be considered mimics of nakijiquinones. Possible advantage of this approach would be to afford derivatives with better solubility in water, and a better

selectivity than avarone, since it is expected that the electronic and steric effects of the amino acid substituent would decrease the reactivity of the quinone moiety with cellular nucleophiles. Different tautomeric forms of the products could have different binding properties with putative targets compared to avarone. Although avarone is the major constituent of the sponge *Dysidea avara*, which has been a subject of cultivation and cell culture projects, availability of large amounts of the compound remains a problem, so it seemed reasonable to investigate whether a simplification of the structure would lead to satisfactory biological activity. Therefore, a very simple model, *tert*-butylquinone was selected as target for modification, with the same amino acids. The model quinone is readily available.

Thus, in this paper, the synthesis and characterization of eighteen new amino acid derivatives of avarone and *tert*-butylquinone are reported. The results of cytotoxic activity investigation of synthesized compounds, against five cancer cell lines and a non-cancerous cell line are also presented. Furthermore, effects of the derivatives on cell cycle analysis of HeLa cells and the caspases activity were analysed. The antibacterial activities of all the compounds against six strains of Gram-positive and four strains of Gram-negative bacteria were determined, as well as the antifungal activity against three fungal species. The biological activity of all derivatives was also examined by the brine shrimp test, *i.e.* toxicity to *Artemia salina*. Electrochemical parameters of all compounds were also determined in order to better understand structure–activity relationships.

2. Results and discussion

2.1. Chemistry

The preparation of the derivatives is shown in **Fig. 2**. The synthesis started with commercially available *tert*-butylhydroquinone or avarol isolated from *D. avara*. The hydroquinones were oxidized using silver oxide to the corresponding quinones. All of the derivatives were obtained by slowly adding an amino acid dissolved in saturated sodium bicarbonate solution, to the ethanol solution of the appropriate quinone, and stirring at room temperature for several hours. Using all amino acids only products arising from substitution in position 3’ were obtained. The exception was L-proline which afforded only 4’-derivatives. In our previous publication [**40**], it was shown that good nucleophiles preferentially add in position

4’ and weaker nucleophiles in position 3’. Proline is much more nucleophilic than the other amino acids [**41**].

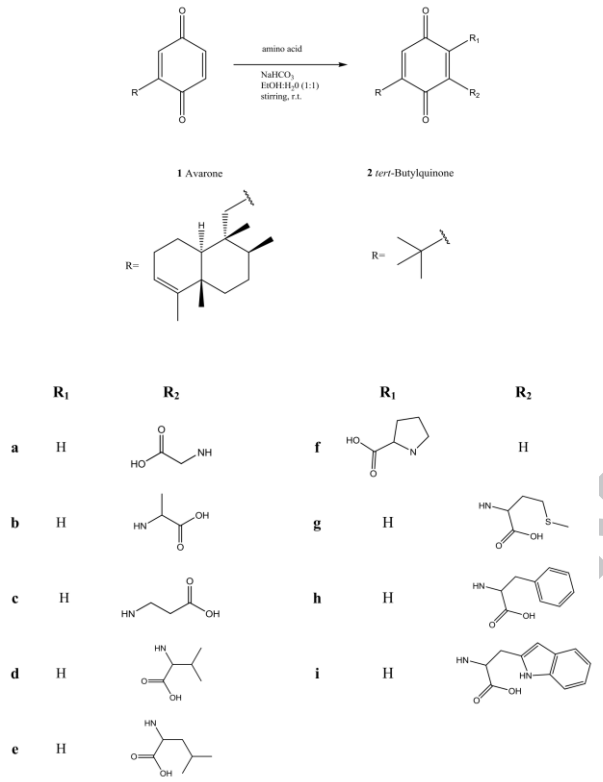


Fig. 2. Chemical structure of avarone and *tert*–butylquinone derivatives

IR spectra, recorded as KBr discs show that the derivatives are in the form of zwitter-ions, based on the absence of carboxylic carbonyl absorption (except for both glycine derivatives), and the presence of the strong carboxylate ion band at 1600–1580 cm^{-1}. The presence of form III (**Fig. 3**) in solid state can be evidenced by a strong C–O vibration band at 1150–1100 cm^{-1}, and a strong broad absorption at 3600–2600 cm^{-1} from superimposed O–H and N–H stretching bands. There are two types of carbonyl stretching vibrations, a stronger band at ca. 1630 cm^{-1} from hydrogen bonded carbonyl group, and a weaker band at 1670 cm^{-1} from a non-hydrogen bonded conjugated carbonyl. The tautomeric equilibrium could be inferred from the severe broadening of signals in NMR spectra taken in $CDCl_3$. However, in CD_3OD the signals are sharp and indicate the dominant presence of quinone tautomeric forms. These forms are now favored since there is no need for formation of a tautomer with a hydroxylic group, because the protic solvent plays the role of the hydrogen bond donor.

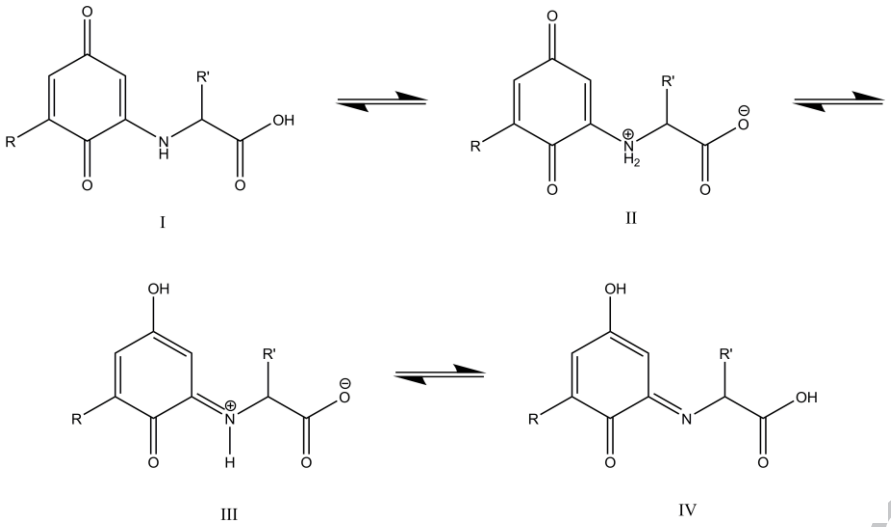


Fig. 3. Tautomeric forms of avarone and t*ert*-butylquinone derivatives

The position of the substituent on the benzoquinone ring was determined using ^{1}H-NMR and ^{13}C-NMR spectroscopy. In the proton spectra, recorded in methanol, of 3'-substituted derivatives signals of quinone protons are doublets with J= 2.2 Hz, while in the spectra of 4'-substituted they are singlets. In ^{13}C NMR spectra the positions of the signals are in accordance with the calculated values [**42**].

Cyclic voltammetry was used for examination of electrochemical properties in order to correlate structure and bioactivity. Cyclic voltammetry measurements of avarone and amino acid derivatives in dimethyl sulfoxide gave two waves corresponding to reversible or quasi-reversible one-electron processes (**Fig. 4**). Such behavior is typical for redox couple quinone/semiquinone anion radical and semiquinone anion radical/hydroquinone dianion, respectively. Amino acid derivatization of avarone led to dramatic shift in potential of the first peak towards more negative potentials (more than 300 mV, **Table 1**). Consequently, the second peak is also shifted cathodically, but less. Generally, derivatization did not alter the reversible behavior of quinone/semiquinone anion radical redox system with peak separation close to ideal values. However, the second peak tended to be broader and/or less pronounced. As a result, semiquinone anion radical/hydroquinone dianion redox system resembles quasi-reversible process with peak separation of more than 80 mV. Destabilization of aminohydroquinone anion radical even led to disappearance of the second reoxidation peak in some cases. Influence of *tert*-butyl substituent followed a trend in which peak potentials were shifted negatively for around 20 mV with respect

to avarone analogues. Such behavior can be easily explained by more positive inductive effect of *tert*-butyl group, and by steric congestion. This effect was more pronounced for the second peak with shift ranging from 30 to 90 mV.

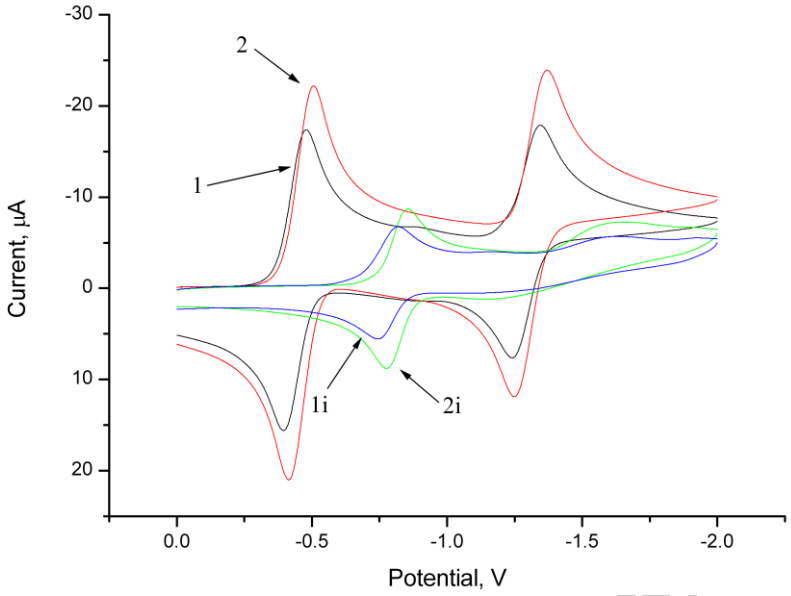


Fig. 4. Cyclic voltammogram of compounds **1**, **1i**, **2** and **2i**

Table 1. The first peak standard potential vs Fc^{+}/Fc couple

Comp.	E_0 (V)	Comp.	E_0 (V)
1	-0.910	**2**	-0.933
1a	-1.187	**2a**	-1.239
1b	-1.244	**2b**	-1.267
1c	-1.226	**2c**	-1.243
1d	-1.261	**2d**	-1.252
1e	-1.265	**2e**	-1.283
1f	-1.362	**2f**	-1.389
1g	-1.239	**2g**	-1.260
1h	-1.256	**2h**	-1.255
1i	-1.256	**2i**	-1.290

2.2. Pharmacology

Cytotoxic activities of investigated compounds against five cancer cell lines: human cervix adenocarcinoma cell line (HeLa), non-small cell lung carcinoma (A549), human melanoma cell (Fem-X), chronic myelogenous leukemia (K562), human breast cancer (MDA-

MB-453) and a non-cancerous cell line, human embryonic lung fibroblast (MRC-5), were determined by MTT assay. The obtained results were expressed as IC_{50} values determined from cell survival diagrams and compared with a widely used anticancer drug cisplatin as positive control. The results are given in **Table 2**. Most compounds showed activity to all five cancer cell lines. The avarone derivatives showed a good cytotoxic activity against all the tested cancer cells lines, and a very weak cytotoxicity to the normal cell line. Derivatives **1c**, **1d** and **1e** were more active than the parent compound against all tested cancer cell lines, with some IC_{50} values in the lower micromolar range. On the other hand, compound **1f** was always less or equally active as avarone. Fem-X cell line was more sensitive for all avarone derivatives (exception is **1f** with a similar value) than avarone.

Table 2. Concentrations of compounds **1-1i** and **2-2i** that induced a 50 % decrease in HeLa, A549, Fem-X, K562, MDA-MB-453 and MRC-5 (expressed as IC_{50} (μM)). All compounds were incubated with cells for 72 h.

Comp.	**HeLa**	**A549**	**Fem-X**	**K562**	**MDA-MB-453**	**MRC-5**
1	18.12 ± 0.68	30.03 ± 2.88	40.33 ± 2.88	17.08 ± 0.35	16.71 ± 1.74	>200
1a	19.87 ± 1.15	46.71 ± 2.53	17.84 ± 1.34	15.63 ± 1.08	5.84 ± 0.92	183.29 ± 1.15
1b	11.66 ± 1.74	54.29 ± 1.01	10.29 ± 1.15	15.75 ± 0.19	14.65 ± 1.62	157.38 ± 2.14
1c	8.87 ± 1.08	26.91 ± 0.25	20.24 ± 2.89	15.47 ± 1.86	3.31 ± 0.71	80.76 ± 1.54
1d	10.92 ± 1.33	23.77 ± 1.21	11.43 ± 0.61	7.78 ± 0.20	8.01 ± 0.57	85.42 ± 1.88
1e	7.72 ± 0.89	19.72 ± 2.83	10.87 ± 0.16	11.17 ± 2.83	10.93 ± 0.51	73.39 ± 1.86
1f	36.23 ± 0.09	49.21 ± 0.75	39.63 ± 1.13	35.44 ± 1.76	60.63 ± 0.06	96.02 ± 2.24
1g	13.91 ± 1.98	47.31 ± 2.86	19.43 ± 1.03	13.58 ± 1.72	25.58 ± 1.20	>200
1h	14.64 ± 1.16	40.08 ± 3.43	17.26 ± 1.59	20.46 ± 0.87	16.55 ± 0.93	>200
1i	16.24 ± 2.29	39.56 ± 2.21	14.82 ± 2.22	16.12 ± 0.29	22.94 ± 1.82	157.39 ± 2.19
2	44.08 ± 2.11	47.02 ± 1.27	41.64 ± 2.55	15.54 ± 1.48	56.83 ± 2.07	>200
2a	131.67 ± 2.76	>200	34.33 ± 0.84	85.02 ± 1.61	171.26 ± 2.35	>200
2b	89.12 ± 4.09	>200	20.11 ± 1.53	77.63 ± 2.38	112.24 ± 1.73	>200
2c	47.11 ± 3.22	171.21 ± 3.27	28.42 ± 0.95	66.96 ± 2.42	94.09 ± 1.23	>200
2d	96.55 ± 1.08	154.47 ± 0.75	24.55 ± 0.34	57.84 ± 1.57	133.25 ± 0.55	>200
2e	57.22 ± 0.34	97.93 ± 0.42	39.37 ± 2.32	76.54 ± 1.91	84.89 ± 0.43	>200
2f	44.94 ± 0.69	186.03 ± 2.47	87.30 ± 1.31	49.24 ± 2.69	67.17 ± 1.04	>200
2g	77.41 ± 0.24	>200	34.82 ± 1.73	88.51 ± 1.33	150.95 ± 2.57	>200
2h	66.31 ± 1.52	164.17 ± 3.51	67.29 ± 1.11	82.26 ± 2.36	68.01 ± 1.18	>200
2i	106.31 ± 1.77	198.04 ± 1.21	34.71 ± 1.12	25.04 ± 3.23	119.86 ± 1.27	>200
Cisplatin	2.1 ± 0.20	11.92 ± 2.19	4.71 ± 0.20	6.89 ± 0.21	3.48 ± 0.22	14.21 ± 1.54

Compound **1c** showed the highest activity against the human breast cancer cell line, MDA-MB-453, with IC_{50} value similar to that for cisplatin. It should be pointed out that **1c** unlike cisplatin showed a significantly lower cytotoxicity against MRC-5 cell line. The best selectivity was observed for **1a** against the MDA-MB-453 cell line. Compound **1a** was almost thirteen times less active against the non-tumor cell line than the positive control.

Only some structure-activity relations could be established. The activity was generally enhanced by introducing both small (including β-alanine) and voluminous amino acids with aliphatic hydrocarbon side chain.

Aromatic side chain did not improve the activity and proline residue decreased both the activity and the selectivity. Glycine and β-alanine derivatives were selective for the breast cancer cell line, while alanine, valine, leucine and isoleucine derivatives showed a high activity, but without a pronounced specificity among the tumor cell lines, although they displayed an excellent selectivity for tumor cell lines compared to the normal MRC-5 cell line.

Electrochemical results have shown that introduction of amino acid substituents did not significantly change redox properties, *i.e.* the quinones still underwent two one–electron reduction processes, with semiquinone intermediates. The reduction potentials for all derivatives are more negative than for the parent compound, indicating a more favourable reoxidation of semiquinone radicals with oxygen, generating reactive superoxide radical which can subsequently produce other reactive oxygen species. However, the differences in activity cannot be ascribed to redox properties, since the redox potentials do not differ within this series of compounds. Even the lower activity of the proline derivative is probably not a consequence of its more negative redox potential, since the whole set of compounds have a lower redox potential and mostly a higher cytotoxicity than the parent compound.

The derivatives of the avarone mimic, *tert*-butylquinone, although non-cytotoxic to normal cells showed a much lower activity than the corresponding avarone derivatives. Structure–activity relationships could not be established within this series of compounds.

The results can be compared with those of some 4'-amino acid avarone derivatives. 4'-Alanino, 4'-phenylalanino and 4'-leucino avarone derivatives showed a strong activity to L1210 mouse leukemia cells (IC_{50} ca.10 μM), however without selectivity in comparison to normal cells [**43**]. Cytotoxic activity of 4'-leucinoavarone was recently studied in more details [**44,45**]. The panel of seven tumor cell lines and one normal cell line was used, and the IC_{50} values from 4 to 17 μM were obtained for tumor cells and >100 μM for normal cell line MRC-5. The results for three cell lines can be compared to those of the regioisomeric 3'-leucino derivative presented in this paper. The activities were similar – 4'-derivative was slightly more active to A549 cells, and 3'-derivative to HeLa cells. In another recent publication cytotoxicity of amino acid derivatives of 1,4–naphthoquinone was assayed against a panel of tumor cells, and normal PBMC (peripheral blood mononuclear cells) [**46**]. For this type of compounds, glycine, alanine and phenylalanine derivatives showed a better activity than β-alanine and proline ones. The best selectivity with respect to normal cells was attained with phenylalanine derivatives.

Given that compounds **1c-e** and **1g** showed the highest cytotoxicity towards HeLa cells, these compounds were selected for examination of the mechanism of action by cytofluorimetric analysis, using propidium iodide to label DNA. Based on the results of MTT test, the IC_{50} and $2IC_{50}$ concentrations of compounds were used to verify cell cycle arrest. **Figs 5** and **6** show the cell-cycle distribution of HeLa cells incubated in the absence or presence of compounds for 24 hours, the approximate doubling time of this cell line. The results indicate an alteration in the percentage of cells in each stage of the cell cycle: sub-G1, G1, S and G2/M, as compared to the control. For **1d a**nd **1e** at applied concentrations a significant increase in the number of cells in the sub-G1 phase and a corresponding decrease in the G1 phase were observed as compared to the control cells. In contrast, compounds **1c** and **1g** caused G2/M arrest after 24 h of treatment. For compound **1d** sub-G1 accumulation was accompanied by a slightly heightened G2/M cell cycle blockade. In addition, as shown in **Figs 5** and **6**, the sub-G1 and G2/M arrest was detected in a concentration-dependent manner. The present study suggests that the cell cycle arrest and induction of apoptosis might be one possible mechanism of action of these compounds in human cancer cells. The greatest effect, both in cytotoxicity and in cell cycle perturbation and induction of apoptosis was achieved with avarone derivatives with branched amino acid chains.

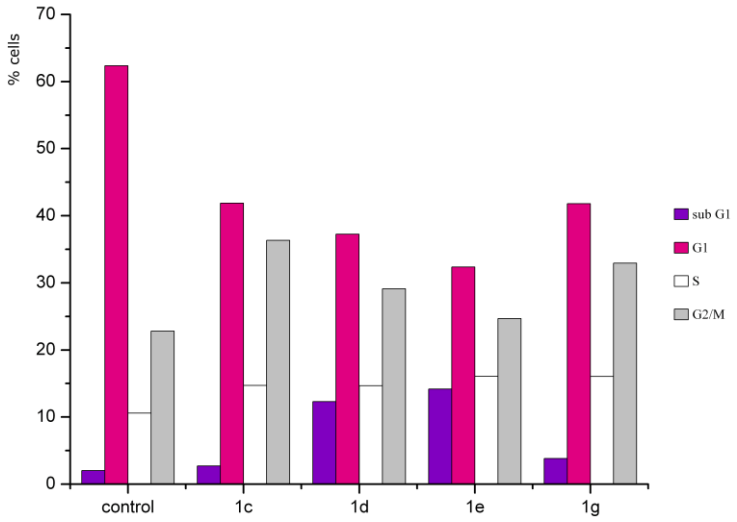


Fig. 5. Cell cycle distribution after 24 h continuous action of investigated compounds. Concentration corresponded to IC_{50}.

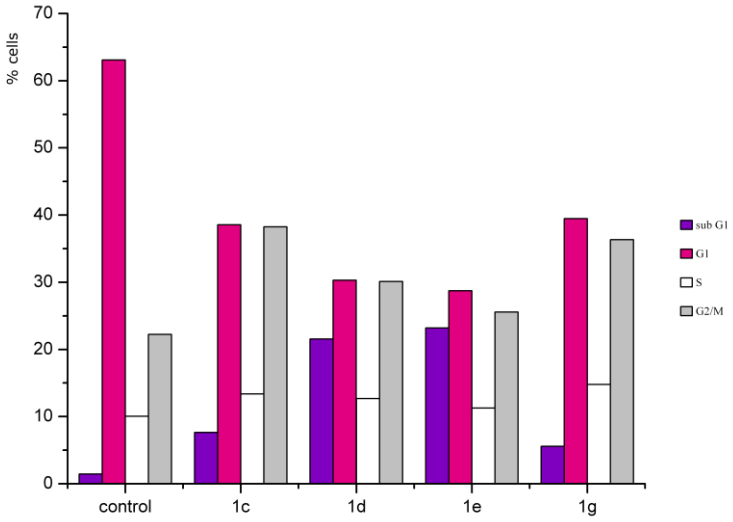


Fig. 6. Cell cycle distribution after 24 h continuous action of investigated compounds. Concentration corresponded to $2IC_{50}$.

To examine the mechanism of apoptosis induced by the investigated compounds in HeLa cells, caspase activities for two most active compounds **1c** and **1e** were measured using specific caspase inhibitors. The activity of caspases 3, 8 and 9, was evaluated after 24 h of incubation with **1c** or **1e** (**Fig. 7**).

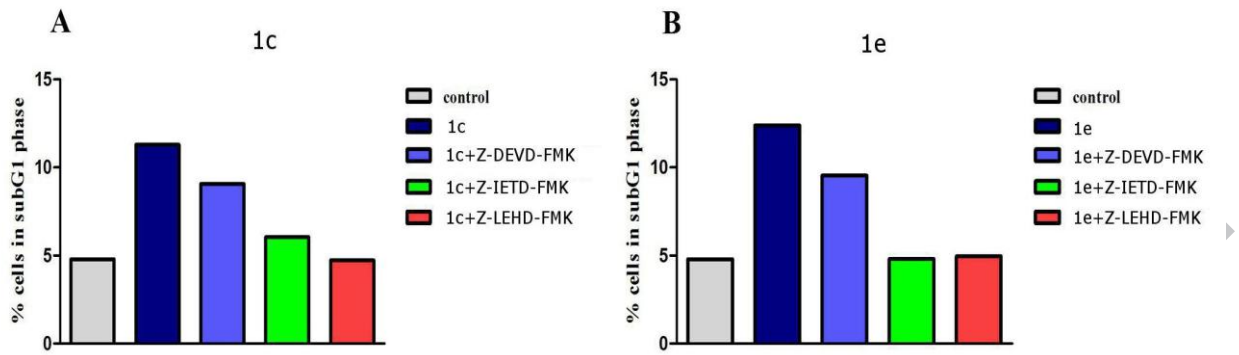


Fig. 7. Influence of compounds **1c (A)** and **1e (B)** alone or in combination with inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHD-FMK) on the fraction of HeLa cells in sub-G1 phase.

The results show that the apoptosis induced by compounds **1c** and **1e** was mediated by stimulating caspase-3, -8 and -9 activities. It is evident that specific inhibitors significantly suppressed the caspase activities and increased the cell viability. In addition, the inhibitor of caspase-3 shows a much weaker influence on the activity of the investigated compounds, suggesting the induction of apoptotic cell death mainly via mitochondrial pathway through activation of caspases 8 and 9 in HeLa cells. Generally, the activation of caspases 3, 8 and 9 might be involved in apoptosis induced by compounds in HeLa cells.

Antibacterial activities of all the compounds against six strains Gram-positive and four Gram-negative bacteria were determined as MIC values (μM) (**Table 3**). Generally, avarone compounds demonstrated significantly stronger activities than *tert*-butylquinone derivatives. All avarone derivatives had MIC values below or similar to 100 μM against *Staphylococcus aureus*, *Micrococcus luteus* and *Escherichia coli*. The weakest activity was shown against the Gram-positive bacteria *Kocuria rhizophila*. Antibacterial effect of the compound **1e** to *S. aureus* and *E. coli* was the most pronounced. Its MIC values indicate a remarkably stronger antibacterial activity than amikacin, antibiotic used as positive control. Bacterial strain *M. luteus* was also more sensitive to **1e** than amikacin. The results for compounds **1h** and **1i** showed a good activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*, better than amikacin. The compound **1h** showed an excellent activity against *S. aureus* and *M. luteus*, and again higher than that of amikacin. In comparison with avarone, all derivatives showed a stronger activity against *M.*

luteus and *E. coli*. The structure–activity relationships are different from those for cytotoxicity to tumor cells, since the best activity was shown by derivatives with voluminous and aromatic side chains, while small amino acid moieties decreased the activity in comparison to avarone.

Table 3. Antibacterial activity of avarone and *tert*-butylquinone derivatives (MIC values in μM)

Comp.	*S. aureus*	*K. rhizophila*	*B. subtilis*	*M. luteus*	*M. flavus*	*C. sporogenes*	*E. coli*	*P. hauseri*	*S.enterica*	*P. aeruginosa*
1	40.06	20.03	80.13	2000.38	20.03	20.03	440.96	27.56	55.12	13.78
1a	101.04	810.88	406.74	101.04	406.74	406.74	101.04	810.88	406.74	406.74
1b	49.10	1566.42	195.49	97.74	195.49	195.04	49.10	393.48	195.49	195.49
1c	24.53	392.97	392.97	48.81	783.44	392.97	48.81	392.97	783.44	783.44
1d	22.95	1463.70	91.33	22.95	91.33	91.33	22.95	182.67	182.67	91.33
1e	5.55	708.79	88.32	11.10	88.32	44.16	5.55	44.16	88.32	88.32
1f	91.55	734.74	183.10	91.55	183.10	368.54	91.55	183.10	183.10	183.10
1g	42.39	1358.70	169.57	42.39	169.57	169.57	42.39	341.30	169.57	169.57
1h	10.32	330.53	20.63	10.32	82.11	82.11	10.32	82.11	20.63	20.63
1i	19.07	305.45	37.94	19.07	37.94	37.94	19.07	37.94	19.07	37.94
2	237.51	475.03	237.51	237.51	475.03	475.03	237.51	475.03	475.03	475.03
2a	662.45	2637.13	1320.68	662.45	2637.13	2637.13	662.45	2637.13	2637.13	662.45
2b	1247.01	4980.08	2490.04	1247.02	2490.04	2490.04	1247.01	4980.08	2490.04	2490.04
2c	624.80	2487.27	2487.27	1245.62	2487.27	1245.62	1245.62	2487.27	2487.27	2487.27
2d	1121.86	4480.29	2240.14	1121.86	2240.14	2240.14	1121.86	4480.28	4480.28	2240.14
2e	265.88	2130.49	2130.49	265.88	2130.49	2130.49	265.88	2130.49	2130.49	4260.98
2f	566.79	2256.32	1129.96	566.79	2256.32	2256.32	566.79	2256.32	2256.32	2256.32
2g	1006.43	2009.64	2009.65	504.82	2009.64	2009.65	1006.43	2009.65	2009.65	2009.64
2h	480.12	1911.32	1911.32	480.12	1911.32	1911.32	480.12	1911.32	1911.32	1911.32
2i	855.19	1707.65	1707.65	1707.65	1707.65	1707.65	1707.65	1707.65	1707.65	1707.65
Amikacin	18.78	3.42	71.72	13.65	3.42	25.61	8.54	11.95	13.66	85.38

Antifungal activity was evaluated against three fungal species (**Table 4**). All of the avarone derivatives were significantly more active against *Candida albicans* and *Saccharomyces cerevisiae* than the positive control nystatin. Also, almost all of the derivatives showed substantial activity against *Aspergillus brasiliensis*, better than nystatin. The compounds **1d** and **1g** showed more than a thousand times stronger activity against *C. albicans* in comparison to nystatin. Similar effects were produced with **1b** and **1e**. The species *S. cerevisiae* was extremely sensitive to the compound **1i**. *tert*-Butylquinone derivatives were also more active against *C. albicans* and *S. cerevisiae* than the positive control nystatin. However, their activity was again

much weaker than that of avarone derivatives. Besides the effect of the alkyl group bound to the quinone nucleus, no structure–activity correlation could be established.

Table 4. Antifungal activity of avarone and *tert*-butylquinone derivatives (MIC values in μM)

Compound	*C. albicans*	*S.cerevisiae*	*A. brasiliensis*
1	n.a[a]	n.a[a]	n.a[a]
1a	12.60	100.26	403.60
1b	6.13	195.00	1562.50
1c	195.00	12.25	782.50
1d	< 2.88	367.68	164.13
1e	< 5.54	176.47	1414.03
1f	23.01	45.77	734.74
1g	< 2.67	87.78	341.30
1h	164.21	10.32	1315.79
1i	305.45	< 2.39	1215.95
2	121.80	237.51	121.80
2a	662.45	662.45	2637.13
2b	625.50	625.50	1240.01
2c	625.50	310.76	2490.04
2d	562.72	562.72	2240.14
2e	535.84	133.11	1068.26
2f	566.79	566.79	2256.32
2g	504.82	504.82	2009.65
2h	480.12	119.27	957.19
2i	213.11	213.11	1407.65
Nystatin	2700.00	1350.00	1350.00

[a] Not active.

The biological activity of all compounds was examined by the brine shrimp test, *i.e.* toxicity to *Artemia salina* (**Table 5**). The results of this test are reported to show a good qualitative correlation with cancer cell-line cytotoxicity. The avarone derivatives showed a good correlation with antileukemic activity, the most effective compound being **1d**, while **1f** demonstrated the lowest activity. *tert*-Butylquinone compounds, except the parent quinone, were less active in this test, too.

Table 5. Brine shrimp test results of avarone and *tert*-butylquinone derivatives

Compound	LC_{50} (ppm)
1	170.00
1a	487.10
1b	129.40
1c	84.30
1d	50.50
1e	117.00
1f	706.60
1g	147.70
1h	96.70
1i	227.40
2	27.40
2a	197.40
2b	393.80
2c	833.20
2d	574.90
2e	104.80
2f	883.40
2g	360.20
2h	196.80
2i	334.10

3. Conclusion

In summary, nine amino acid derivatives of marine quinone avarone (**1**) and the same number of derivatives of model system *tert*-butylquinone (**2**) were obtained. Cytotoxic activities of synthesized compounds towards five cancer cell lines: HeLa, A549, Fem-X, K562, MDA-MB-453 and a non-cancerous cell line MRC-5 were determined by MTT assay. Most tested avarone derivatives had a good antitumor activity with IC_{50} values lower than for parent compound. The strongest cytotoxic activity, similar to widely used drug cisplatin, was displayed by derivative **1c** (IC_{50} 3.31 μM against MDA-MB-453). Towards HeLa cells, compounds **1b-e** and **1g** showed the highest cytotoxicity. These derivatives were chosen for investigation of cell cycle by cytofluorimetric analysis. The tested compounds showed different effects on cell cycle. Compounds **1d** and **1e** led to an increase in a number of cells in the sub-G1 phase while derivatives **1c** and **1g** caused G2/M arrest. The obtained results show that apoptosis might be the most likely mechanism of action of investigated compounds. Further research of the mechanism

of apoptosis was evaluated using specific caspase inhibitors. The results suggested that derivatives **1c** and **1e** activated caspases 8 and 9, and that cell death occurred via mitochondrial pathway.

The synthesized compounds were tested toward a panel of Gram positive and Gram negative bacteria, as well as several fungi strains. Some of derivatives showed a significant antimicrobial activity, especially avarone agents against *C. albicans* and *S. cerevisiae*, while parent compound was not active.

4. Experimental protocols

4. 1. Chemistry

Melting points (°C, uncorrected) were determined on a Boetius PHMK apparatus (VEB Analytic, Dresden, Germany). ^{1}H and ^{13}C NMR spectra were recorded at 200 MHz and 50 MHz, respectively on Varian YH 200 instrument, in deuterated methanol using tetramethylsilane as an internal standard. Numeration scheme is given in the Supplementary material. High resolution mass spectra were taken on Mass Spectrometer 6210 Time of Fligth LC-MS system, Agilent Technologies. UV/Vis spectra were recorded on a Cintra 40 UV-Visible spectrometer. Polarimetric measurements were performed on a Rudolph research analytical polarimeter, Autopol IV, in methanol. IR spectra were recorded on Thermo Scientific Nicolet 6700 FT-IR (Smart orbit).

Cyclic voltammetry experiments were performed at room temperature under nitrogen atmosphere in a three-electrode cell at 25 ºC and using a CHI760B workstation (CH Instruments, Austin TX, USA). The working electrode was glassy carbon disk (3 mm diameter). The counter electrode was a platinum wire, and a silver wire immersed in electrolyte solution containing 0.01 M silver ions was used as the reference electrode. The quinone derivatives were used as 2 mM solution in dimethyl sulfoxide, with 0.1 M tetraethylammonium perchlorate as an electrolyte.

Avarol was isolated from the sponge *Dysidea avara* collected in the Bay of Kotor, Montenegro, and oxidized with silver oxide in order to obtain avarone as described above.

All reagents used were commercial products purchased from Merck and Sigma-Aldrich. Column chromatography was carried out on silica gel (0.063-0.200 mm, Merck). Analytical thin-

layer chromatography (TLC) was performed on precoated aluminum-backed plates (silica gel 60 F254, Fluka).

4.1.1. Synthesis of derivatives, general procedure

4.1.1.1. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2.5-dioxo-1,4-cyclohexadien-1-yl]glycine (**1a**)

Glycine (240 mg) was dissolved in 100 ml of saturated solution of $NaHCO_3$, and a few drops of aqueous solution of NaOH until pH 10 was added. Solution was added in portions to the 100 ml of ethanol solution of avarone (200 mg) and the color changed from yellow to red. The reaction mixture was stirred for 4.5 h at room temperature. After ethanol was eliminated, the remaining aqueous solution was extracted with ethyl acetate and the solution was dried over anhydrous Na_2SO_4. After the evaporation of ethyl acetate, the product was purified by preparative TLC (silica gel GF_{254}, 1 mm) using chloroform/methanol (9:1) as the eluent, and re-chromatographed with the same eluent. Compound **1a** was obtained after recrystallization in dichlormethane overnight as red crystals (107 mg; yield 43 %). Mp: 86–88 ºC; $[\alpha]_D^{20}$ = –16 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.33 (d, J= 1.6 Hz, 1H, **C6'-H**), 5.32 (d, J= 1.6 Hz, 1H, **C4'-H**), 5.09 (brs, 1H, **C3-H**), 3.62 (s, 2H, **C7'-H**), 2.57 (d, J= 13.4 Hz, 1H, **C11- H_a**), 2.45 (d, J= 13.4 Hz, 1H, **C11- H_b**), 1.49 (s, 3H, **C12-H**), 0.98 (s, 3H, **C13-H**), 0.93 (d, J=6.2 Hz, 3H, **C14-H**), 0.82 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.1 (**C5'**), 184.5 (**C2'**), 174.6 (**C8'**), 148.6 (**C3'**), 144.9 (**C4**), 143.8 (**C1'**), 140.8 (**C6'**), 121.6 (**C3**), 97.6 (**C4'**), signal overlapped with solvent signal (**C10**), 47.0 (**C7'**), 43.0 (**C9**), 39.4 (**C5**), 38.0 (**C8**), 37.3 (**C6**), 36.1 (**C11**), 28.6 (**C7**), 27.3 (**C2**), 20.5 (**C1**), 20.3 (**C13**), 18.4 (**C14**), 18.1 (**C12**), 17.2 (**C15**); IR (KBr, cm^{-1}**):** 3456.7, 3330.3, 2959.4, 2930.5, 2860.9, 1725.4, 1673.9, 1636.4, 1593.8, 1457.8, 1382.0, 1352.7, 1262.6, 1125.8, 1073.6, 739.1**;** CIMS (MeOH): 386.23150 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 286 (4.73), 482 (4.20).

4.1.1.2. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)alanine (**1b**)

Obtained by the same procedure as **1a**, using 200 mg avarone and 179 mg L-alanine, but without addition of aqueous solution of NaOH. The reaction time was shortened to 3.5 h and reaction mixture was extracted with chloroform. The crude product was chromatographed on a

silica gel 60 column, using chloroform/methanol (8:2) as the eluent, and rechromatographed by preparative TLC (silica gel GF_{254}, 1 mm, eluent chloroform/methanol (8:2)), affording **1b** as red crystals (65 mg; yield 25 %). Mp: 168–171 ºC (dec.); $[\alpha]_D^{20}$ = 56 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.24 (d, J= 2.4 Hz, 1H, **C6'-H**), 5.27 (d, J=2.4 Hz, 1H, **C4'-H**), 5.02 (brs, 1H, **C3-H**), 3.71 (q, J=6.8 Hz, 1H, **C7'-H**), 2.48 (d, J= 6.2Hz, 1H, **C11- H_a**), 2.41 (d, J= 6.2 Hz, 1H, **C11- H_b**), 1.42 (s, 3H, **C12-H**), 1.33 (d, J= 6.8 Hz, 3H, **C9'-H**), 0.92 (s, 3H, **C13-H**), 0.86 (d, J= 6.0 Hz, 3H, **C14-H**), 0.76 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.2 (**C5'**), 184.7 (**C2'**), 178.7 (**C8'**), 148.1 (**C3'**), 145.2 (**C4**), 144.0 (**C1'**), 140.7 (**C6'**), 121.6 (**C3**), 97.7 (**C4'**), 54.1 (**C7'**), signal overlapped with solvent signal (**C10**), 43.1 (**C9**), 39.6 (**C5**), 38.0 (**C8**), 37.4 (**C6**), 36.1 (**C11**), 28.6 (**C7**), 27.4 (**C2**), 20.5 (**C1**), 20.4 (**C13**), 18.3 (**C14**), 18.1 (**C12**), 17.3 (**C9'**), 17.2 (**C15**); IR (KBr, cm^{-1}**):** 3363.9, 2960.8, 2931.4, 2855.5, 1670.7, 1633.0, 1583.0, 1498.8, 1455.0, 1416.4, 1358.7, 1335.0; CIMS (MeOH): 400.24715 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 290 (4.65), 492 (4.26).

4.1.1.3. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]β-alanine (**1c**)

Obtained by the same procedure as **1a**, using 100 mg avarone and 143 mg β-alanine. The reaction time was shortened to 3 h. Rechromatography was not necessary. **1c**: red crystals; 43 mg (yield 34 %). Mp: 120–122 ºC; $[\alpha]_D^{20}$ = 64 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.32 (d, J= 2.8 Hz, 1H, **C6'-H**), 5.46 (d, J= 2.8Hz, 1H, **C4'-H**), 5.11 (brs, 1H, **C3-H**), 3.34 (t, J= 5.2 Hz, 2H, **C7'-H**), 2.60 (d, J= 5 Hz, 1H, **C11- H_a**), 2.53 (d, J= 5 Hz, 1H, **C11- H_b**), 2.45 (t, J= 6.6 Hz, 2H, **C8'-H**), 1.51 (s, 3H, **C12-H**), 1.00 (s, 3H, **C13-H**), 0.93 (d, J=6.2 Hz, 3H, **C14-H**), 0.83 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.1 (**C5'**), 184.7 (**C2'**), 179.5 (**C9'**), 149.4 (**C3'**), 145.0 (**C4**), 143.9 (**C1'**), 140.7 (**C6'**), 121.6 (**C3**), 97.0 (**C4'**), signal overlapped with solvent signal (**C10**), 43.0 (**C9**), 40.8 (**C7'**), 39.5 (**C5**), 38.1 (**C8**), 37.4 (**C6**), 36.7 (**C8'**), 36.1 (**C11**), 28.6 (**C7**), 27.4 (**C2**), 20.5 (**C1**), 20.3 (**C13**), 18.3 (**C14**), 18.1 (**C12**), 17.2 (**C15**); IR (KBr, cm^{-1}**):** 3386.4, 2960.3, 2858.0, 1671.2, 1633.3, 1583.1, 1513.4, 1451.4, 1382.3, 1254.3, 1143.6, 1030.4; CIMS (MeOH): 400.24846 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 290 (4.75), 490 (4.33).

4.1.1.4. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*R*)valine (**1d**)

Obtained by the same procedure as **1a**, using 100 mg avarone and 113 mg D-valine. The reaction time was shortened to 3 h and after evaporation of ethyl acetate the product was purified by preparative TLC (silica gel GF_{254}, 1 mm, eluent toluene/ethyl acetate (9:1)). **1d**: red crystals; 55 mg (yield 40 %). Mp: 197–200 ºC; $[\alpha]_D^{20}$ = –36 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.33 (d, J= 2.8 Hz, 1H, **C6'-H**), 5.42 (d, J= 2.8 Hz, 1H, **C4'-H**), 5.11 (brs, 1H, **C3-H**), 3.60 (d, J= 4.6 Hz, 1H, **C7'-H**), 2.59 (d, J= 14 Hz, 1H, **C11- H_a**), 2.48 (d, J= 14 Hz, 1H, **C11- H_b**), 2.19 (m, 1H, **C9'-H**), 1.51 (s, 3H, **C12-H**), 1.07 (d, J= 1.6 Hz, 3H, **C10'-H**), 1.01 (s, 3H, **C13-H**), 0.99 (d, J= 1.6 Hz, 3H, **C11'-H**), 0.96 (d, J= 2.2 Hz, 3H, **C14-H**), 0.85 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.3 (**C5'**), 184.7 (**C2'**), 177.6 (**C8'**), 148.7 (**C3'**), 145.0 (**C4**), 143.9 (**C1'**), 140.7 (**C6'**), 121.7 (**C3**), 97.7 (**C4'**), 64.7 (**C7'**), signal overlapped with solvent signal (**C10**), 43.1 (**C9**), 39.6 (**C5**), 38.3 (**C8**), 37.4 (**C6**), 36.3 (**C11**), 32.4 (**C9'**), 28.6 (**C7**), 27.4 (**C2**), 20.5 (**C1, C11'**), 19.8 (**C13**), 19.2 (**C14**), 18.3 (**C10'**), 18.0 (**C12**), 17.2 (**C15**); IR (KBr, cm^{-1}**):** 3356.7, 2961.7, 2931.1, 2875.4, 1670.7, 1632.8, 1580.2, 1507.5, 1417.7, 1384.8, 1344.5, 1288.5, 1124.4, 1092.5, 644.2, 612.2; CIMS (MeOH): 428.27917 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 286 (4.68), 498 (4.34).

4.1.1.5. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)leucine (**1e**)

Obtained by the same procedure as **1a**, but the reaction time was prolonged to 5 h and the crude product was first purified by preparative TLC (silica gel GF_{254}, 1 mm, eluent toluene/ethyl acetate (9:1)). Starting from 100 mg avarone and 126 mg L-leucine, **1e** was obtained as red crystals (42 mg; yield 30 %). Mp: 148–150 ºC; $[\alpha]_D^{20}$ = –24 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.32 (d, J= 2.8 Hz, 1H, **C6'-H**), 5.39 (d, J=2.8 Hz, 1H, **C4'-H**), 5.103 (brs, 1H, **C3-H**), 3.77 (t, J= 5.6 Hz, 1H, **C7'-H**), 2.52 (d, J= 14.0 Hz, 1H, **C11- H_a**), 2.46 (d, J= 14 Hz, 1H, **C11- H_b**), 1.71 (m, 3H, **C9'**, **C10'**), 1.51 (s, 3H, **C12-H**), 1.01 (s, 3H, **C13-H**), 0.89 (d, J= 6.2 Hz, 3H, **C14-H**), 0.96 (s, 3H, **C11'**), 0.93 (s, 3H, **C12'**), 0.84 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.2 (**C5'**), 184.7 (**C2'**), 178.2 (**C8'**), 148.6 (**C3'**), 145.2 (**C4**), 144.0 (**C1'**), 140.6 (**C6'**), 121.5 (**C3**), 97.5 (**C4'**), 58.0 (**C7'**), signal overlapped with solvent signal (**C10**), 43.1 (**C9**), 42.0 (**C9'**), 39.6 (**C5**), 37.8 (**C8**), 37.4 (**C6**), 36.0 (**C11**), 28.6 (**C7**), 27.4 (**C2**), 26.4 (**C10'**), 23.5 (**C11'**), 22.8 (**C12'**), 20.5 (**C1**), 20.3 (**C13**), 18.3 (**C14**), 18.1 (**C12**), 17.2 (**C15**); IR (KBr, cm^{-1}**):** 3368.4, 1670.0, 1632.7, 1581.0, 1512.1, 1449.6, 1384.4, 1343.1, 1257.4, 1121.1,

1029.4, 737.1, 702.6, 613.5; CIMS (MeOH): 442.29476 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 286 (4.62), 494 (4.24).

4.1.1.6. *N*-[4-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)proline (**1f**)

Obtained by the same procedure as **1a**, using 110 mg avarone and 122 mg L-proline, but the color changed from yellow to purple. The reaction time was shortened to 1.5 h and the product was purified by preparative TLC (silica gel GF_{254}, eluent toluene/ethyl acetate (9:1)), then rechromatographed with the chloroform/methanol (8:2) as the eluent. Compound **1f** was obtained as red-purple crystals (28 mg; yield 19 %). Mp: 193–195 °C (dec.); $[\alpha]_D^{20}$ = –120 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.13 (brs, 1H, **C6'-H**), 5.37 (brs, 1H, **C3'-H**), 5.00 (bs, 1H, **C3-H**), 3.57-3.42 (m, 3H, **C7'-H**, **C10'-H**), 2.51 (d, J= 13.0 Hz, 1H, **C11- H_a**), 2.38 (d, J= 13.0 Hz, 1H, **C11- H_b**), 2.19-2.05 (m, 2H, **C9'-H**), 2.05-1.85 (broad signal, 2H, **C8'-H**), 1.41 (s, 3H, **C12-H**), 0.91 (s, 3H, **C13-H**), 0.85 (d, J= 6.2 Hz, 3H, **C14-H**), 0.75 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 186.3 (**C2'**), 185.7 (**C5'**), 180.0 (**C11'**), 150.1 (**C1'**), 148.9 (**C4'**), 144.9 (**C4**), 134.5 (**C6'**), 121.7 (**C3**), 102.5 (**C3'**), 66.9 (**C10'**), signal overlapped with solvent signal (**C10**), 43.7 (**C9**), 39.5 (**C5**), 38.2 (**C8**), 37.4 (**C6**), 36.1 (**C11**), 33.0 (**C9'**), 30.7 (**C8'**), 28.7 (**C7**), 27.4 (**C2**), 20.5 (**C1**), 20.4 (**C13**), 18.3 (**C14**), 18.2 (**C12**), 17.3 (**C15**); IR (KBr, cm^{-1}**):** 3377.8, 2959.8, 2925.5, 2876.4, 1658.7, 1629.5, 1590.2, 1563.9, 1452.0, 1420.8, 1144.6; CIMS (MeOH): 426.26373 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 296 (4.67), 512 (4.34).

4.1.1.7. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)methionine (**1g**)

Obtained by the same procedure as **1a**, using 200 mg avarone and 239 mg L-methionine. The reaction time was prolonged to 5 h. The product was purified using toluene/ethyl acetate (9:1) as the eluent and then rechromatographed with the eluent chloroform/methanol (8:2). **1g**: red crystals; 45 mg (yield 15 %). Mp: 164–168 °C; $[\alpha]_D^{20}$ = –12 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.33 (d, J= 2.2 Hz, 1H, **C6'-H**), 5.45 (d, J= 2.2 Hz, 1H, **C3'-H**), 5.10 (brs, 1H, **C3-H**), 3.89 (dd, J= 5.6 Hz, J= 9.0 Hz, 1H, **C7'-H**), 2.66-2.37 (m, 4H, **C11- H_a**, **C11- H_b**, **C10'-H**), 2.17-2.10 (m, 2H, **C9'-H**), 2.04 (s, 3H, **C11'-H**), 1.50 (s, 3H, **C12-H**), 1.00 (s, 3H,

C13-H), 0.94 (d, J= 6.2 Hz, 3H, **C14-H**), 0.84 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.2 (**C5'**), 184.6 (**C2'**), 176.9 (**C8'**), 148.3 (**C3'**), 145.1 (**C4**), 144.1 (**C1'**), 140.6 (**C6'**), 121.5 (**C3**), 97.7 (**C4'**), 57.9 (**C7'**), signal overlapped with solvent signal (**C10**), 43.1 (**C9**), 39.5 (**C5**), 37.8 (**C8**), 37.4 (**C6**), 35.9 (**C11**), 32.1 (**C9'**), 31.1 (**C10'**), 28.5 (**C7**), 27.4 (**C2**), 20.5 (**C1**), 20.3 (**C13**), 18.3 (**C14**), 18.1 (**C12**), 17.1 (**C15**), 15.4 (**C11'**); IR (KBr, cm^{-1}**):** 3350.0, 2959.2, 2931.1, 2857.9, 1669.7, 1632.1, 1584.4, 1489.0, 1442.6, 1384.6, 1339.1, 1284.1, 1144.8, 1029.6, 628.8; CIMS (MeOH): 460.25091 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 290 (4.59), 490 (4.20).

4.1.1.8. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)phenylalanine (**1h**)

Obtained by the same procedure as **1a**, using 190 mg avarone and 100 mg L-phenylalanine and the reaction time was prolonged to 5 h. After the evaporation of ethyl acetate, the product was purified by preparative TLC (silica gel GF_{254}, 1 mm, eluent toluene/ethyl acetate (9:1), affording **1h** as red crystals (28 mg; yield 10 %). Mp: 155–158 ºC; $[\alpha]_D^{20}$ = 60 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 7.20-7.08 (broad signal, 5H, **Ph-H**), 6.26 (d, J= 2.2 Hz, 1H, **C6'-H**), 5.21 (d, J= 2.2 Hz, 1H, **C4'-H**), 5.13 (brs, 1H, **C3-H**), 3.98 (dd, J= 4.4 Hz, J= 7.8 Hz, 1H, **C7'-H**), 3.27 (dd, J= 6.0 Hz, J= 8.0 Hz, 1H, **C9'-H_a**), 2.99 (dd, J= 8.0 Hz, J= 14.0 Hz, 1H, **C9'-H_b**), 2.66 (d, J= 13.4 Hz, 1H, **C11- H_a**), 2.30 (d, J= 14.0 Hz, 1H, **C11- H_b**), 1.54 (s, 3H, **C12-H**), 1.01 (s, 3H, **C13-H**), 0.92 (d, J= 6.2 Hz, 3H, **C14-H**), 0.82 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.1 (**C5'**), 184.3 (**C2'**), 176.8 (**C8'**), 148.0 (**C3'**), 145.0 (**C4**), 143.8 (**C1'**), 140.5 (**C6'**), 138.7 (**C10'**), 130.5 (**C11'**, **C15'**), 129.4 (**C12'**, **C14'**), 127.6 (**C13'**), 121.8 (**C3**), 97.5 (**C4'**), 59.9 (**C7'**), signal overlapped with solvent signal (**C10**), 43.0 (**C9**), 39.5 (**C5**), 39.0 (**C9'**), 37.5 (**C8**), 37.4 (**C6**), 35.6 (**C11**), 28.4 (**C7**), 27.5 (**C2**), 20.5 (**C1**), 20.3 (**C13**), 18.4 (**C14**), 18.1 (**C12**), 17.0 (**C15**); IR (KBr, cm^{-1}**):** 3365.2, 3026.4, 2957.0, 2929.6, 2857.3, 1670.0, 1631.9, 1581.7, 1514.3, 1494.2, 1440.9, 1409.8, 1341.5, 1288.9, 1127.5, 1028.9; CIMS (MeOH): 476.27878 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 286 (4.66), 496 (4.26).

4.1.1.9. *N*-[3-[[(1R,2S,4aS,8aS)-1,2,3,4,4a,7,8,8a-octahydro-1,2,4a,5-tetramethyl-1-naphthalenyl)methyl]-2,5-dioxo-1,4-cyclohexadien-1-yl]-(*S*)tryptophan (**1i**)

Obtained by the same procedure as **1a**, using 100 mg avarone and 98 mg L-tryptophan, but the color changed from yellow to red-purple and the reaction time was shortened to 3 h. For

the first preparative TLC, eluent toluene/ethyl acetate (9:1) was used. **1i**: red crystals; 52 mg (yield 32 %). Mp: 157–160 ºC (dec.); $[\alpha]_D^{20}$ = –32 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 7.55 (d, J= 7.2 Hz, 1H, **C16'-H**), 7.30 (d, J= 8.0 Hz, 1H, **C13'-H**), 7.09 (s, 1H, **C11'-H**), 7.05-6.92 (m, 2H, **C14'-H**, **C15'-H**), 6.23 (brs, 1H, **C6'-H**), 5.27 (brs, 1H, **C4'-H**), 5.07 (brs, 1H, **C3-H**), 4.09 (brs, 1H, **C7'-H**), 3.55-3.40 (brm, 1H, **C9'-H_a**), 3.30-3.15 (brm, 1H, **C9'-H_b**), 2.55 (d, J= 13.4 Hz, 1H, **C11- H_a**), 2.28 (d, J= 13.4 Hz, 1H, **C11- H_b**), 1.52 (s, 3H, **C12-H**), 0.99 (s, 3H, **C13-H**), 0.88 (d, J= 6.2 Hz, 3H, **C14-H**), 0.80 (s, 3H, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.0 (**C5'**), 184.1 (**C2'**), 179.8 (**C8'**), 148.1 (**C3'**), 144.5 (**C4**), 143.4 (**C1'**), 140.4 (**C6'**), 137.7 (**C12'**), 128.8 (**C17'**), 124.3 (**C11'**), 122.2 (**C16'**), 121.7 (**C3**), 119.8 (**C15'**), 119.2 (**C14'**), 112.1 (**C13'**), 111.2 (**C10'**), 97.5 (**C4'**), 54.5 (**C7'**), signal overlapped with solvent signal (**C10**), 42.8 (**C9**), 39.3 (**C5**), 37.4 (**C8**), 37.0 (**C6**), 35.7 (**C11**), 28.8 (**C9'**), 28.3 (**C7**), 27.2 (**C2**), 20.4 (**C1**), 20.1 (**C13**), 18.3 (**C14**), 18.1 (**C12**), 17.0 (**C15**); IR (KBr, cm^{-1}**):** 3351.8, 2956.1, 2839.6, 1664.8, 1632.6, 1581.7, 1439.9, 1412.7, 1145.3, 1024.0, 740.7, 659.7, 637.6, 611.3; CIMS (MeOH): 515.28989 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 282 (4.74), 290 (4.71), 396 (3.82), 500 (4.10).

4.1.1.10. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-glycine (**2a**)

Glycine (324 mg) was dissolved in 100 ml of saturated solution of $NaHCO_3$, and a few drops of aqueous solution of NaOH until pH 10 were added. Solution was added in portions to the 100 ml of ethanol solution of *tert*-butylquinone (180 mg) and the color changed from yellow to red. The reaction mixture was stirred for 2.5h at room temperature. After ethanol had been removed, the remaining aqueous solution was extracted with ethyl acetate. Thereafter aqueous layer was acidified with 10% HCl and again extracted with ethyl acetate. The solutions were dried over anhydrous Na_2SO_4. After the evaporation of ethyl acetate, the product was purified by preparative TLC (silica gel GF_{254}, 1 mm, chloroform/methanol (9:1)) and rechromatographed with the same eluent. The product was recrystallized in dichloromethane overnight, affording **2a** as red crystals (31 mg; yield 12 %). Mp: 104–106 ºC (dec.); ^{1}H NMR (200 MHz, CD_3OD): δ 6.41 (d, J=2.2 Hz, 1H, **C6-H**), 5.30 (d, J= 2.2 Hz, 1H, **C4-H**), 3.64 (brs, 2H, **C11-H**), 1.27 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.8 (**C5**), 183.9 (**C2**), 174.9 (**C12**), 153.1 (**C1**), 149.7 (**C3**), 135.9 (**C6**), 97.0 (**C4**), 47.0 (**C11**), 35.7 (**C7**), 29.4 (**C8**, **C9**, **C10**); IR (KBr, cm^{-1}**):** 3356.9, 2959.9, 2930.6, 2871.1, 1725.8, 1633.2, 1591.1, 1486.1, 1461.1, 1393.9,

1362.5, 1278.0, 1124.1, 1074.2; CIMS (MeOH): 238.10636 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.60), 368 (3.90), 476 (3.98).

4.1.1.11. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-alanine (**2b**)

Obtained by the same procedure as **2a**, using 200 mg *tert*-butylquinone and 170 mg L-alanine, but without adding of aqueous solution of NaOH. The reaction time was prolonged to 3 h. For product purification by preparative TLC (silica gel GF_{254}, 1 mm), chloroform/methanol (8:2) was used as the eluent. Compound **2b** was obtained as red crystals (73 mg; yield 24 %). Mp: 159–163 ºC (dec.); $[\alpha]_D^{20}$ = –32 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.40 (d, J=2.2 Hz, 1H, **C6-H**), 5.35 (d, J= 2.2 Hz, 1H, **C4-H**), 3.81 (q, J= 6.8 Hz, 1H, **C11-H**), 1.43 (d, J= 7.2 Hz, 3H, **C13-H**), 1.26 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.7 (**C5**), 184.0 (**C2**), 179.3 (**C12**), 153.1 (**C1**), 148.8 (**C3**), 135.9 (**C6**), 97.0 (**C4**), 54.0 (**C11**), 35.7 (**C7**), 29.4 (**C8**, **C9**, **C10**), 18.0 (**C13**); IR (KBr, cm^{-1}**):** 3351.9, 2961.3, 1673.1, 1630.3, 1580.8, 1488.0, 1455.2, 1416.4, 1365.3, 1338.6, 1283.0, 1171.5; CIMS (MeOH): 252.12285 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.87), 486 (4.40).

4.1.1.12. *N*-(4-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-β-alanine (**2c**)

Obtained by the same procedure as **2a**, using 100 mg *tert*-butylquinone and 110 mg β-alanine and the reaction time was prolonged to 3 h. Rechromatography was not necessary. **2c**: red crystals; 74 mg (yield 48 %). Mp: 117–120 ºC; ^{1}H NMR (200 MHz, CD_3OD): δ 6.41 (brs, 1H, **C6-H**), 5.44 (brs, 1H, **C4-H**), 3.30 (t, J= 1.6 Hz, 2H, **C11-H**), 2.55 (broad signal, 2H, **C12-H**), 1.25 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.7 (**C5**), 184.0 (**C2**), 178.1 (**C13**), 153.1 (**C1**), 150.1 (**C3**), 135.9 (**C6**), 96.6 (**C4**), 40.4 (**C11**), 35.7 (**C7**), 30.0 (**C12**) 29.4 (**C8**, **C9**, **C10**); IR (KBr, cm^{-1}**):** 3349.5, 2960.8, 1673.4, 1631.6, 1582.2, 1509.9, 1455.5, 1365.4, 1256.1, 1158.3; CIMS (MeOH): 252.12255 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.92), 484 (4.44).

4.1.1.13. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-D-valine (**2d**)

Obtained by the same procedure as **2a**, using 130 mg *tert*-butylquinone and 140 mg D-valine. The crude product was purified by preparative TLC (silica gel GF_{254}, 1 mm) using toluene/ethyl acetate (9:1) as the eluent. Compound **2d** was obtained as red crystals (61 mg; yield 28 %). Mp:

180–183 ºC (dec.); $[\alpha]_D^{20}$ = –20 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.41 (d, J= 2.4 Hz, 1H, **C6-H**), 5.40 (d, J= 2.4 Hz, 1H, **C4-H**), 3.61 (d, J= 6.2 Hz, 1H, **C11-H**), 2.20 (m, 1H, **C13-H**), 1.26 (s, 9H, **C8-H, C9-H, C10-H**), 0.98 (d, J= 6.4 Hz, 6H, **C14-H**, **C15-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.8 (**C5**), 184.0 (**C2**), 177.6 (**C12**), 153.0 (**C1**), 149.6 (**C3**), 135.9 (**C6**), 97.2 (**C4**), 64.5 (**C11**), 35.7 (**C7**), 32.5 (**C13**) 29.4 (**C8**, **C9**, **C10**), 20.0 (**C15**), 19.1 (**C14**); IR (KBr, cm^{-1}**):** 3350.0, 2962.7, 2873.6, 1673.5, 1630.7, 1578.5, 1506.2, 1421.1, 1340.7, 1162.2; CIMS (MeOH): 280.15442 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.78), 490 (4.34).

4.1.1.14. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-leucine (**2e**)

Obtained by the same procedure as **2a**, but the color changed from yellow to red-purple and the reaction time was shortened to 2 h. For the first purification by preparative TLC toluene/ethyl acetate (9:1) was used as the eluent. Starting from 100 mg *tert*-butylquinone and 80 mg L-leucine, **2e** was obtained as red crystals (32 mg; yield 18 %). Mp: 147–148 ºC; $[\alpha]_D^{20}$ = –20 (*c* 0.250, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.32 (d, J= 2.2 Hz, 1H, **C6-H**), 5.29 (d, J= 2.2 Hz, 1H, **C4-H**), 3.68 (m, 1H, **C11-H**), 1.64 (m, 3H, **C13-H**, **C14-H**), 1.18 (s, 9H, **C8-H, C9-H, C10-H**), 0.89 (d, J= 6.0 Hz, 3H, **C15-H**), 0.83 (d, J= 6.0 Hz, 3H, **C16-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.8 (**C5**), 184.0 (**C2**), 178.4 (**C12**), 153.1 (**C1**), 149.4 (**C3**), 135.9 (**C6**), 97.0 (**C4**), 58.0 (**C11**), 42.3 (**C13**), 35.7 (**C7**), 29.4 (**C8**, **C9**, **C10**), 26.4 (**C14**), 23.4 (**C15**), 22.7 (**C16**); IR (KBr, cm^{-1}**):** 3359.8, 2958.5, 2871.7, 1672.3, 1630.8, 1578.7, 1511.2, 1419.7, 1365.0, 1341.6, 1264.7, 1165.1; CIMS (MeOH): 294.16991 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 272 (4.72), 488 (4.258).

4.1.1.15. *N*-(4-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-proline (**2f**)

Obtained by the same procedure as **2a**, using 200 mg *tert*-butylquinone and 280 mg L-proline. The color changed from yellow to red-purple and the reaction time was prolonged to 3 h. The product was rechromatographed by preparative TLC using toluene/ethyl acetate (6:4). **2f**: purple crystals; 29 mg (yield 9 %). Mp: 148–152 ºC (dec.); $[\alpha]_D^{20}$ = –264 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.28 (s, 1H, **C6-H**), 5.40 (s, 1H, **C3-H**), 3.63 (poorly resolved dd overlapping with C15-H, 1H, **C11-H**), 3.55-3.40 (m, 2H, **C15-H**), 2.24-2.12 (m, 2H, **C13-H)**, 1.90 (brs, 2H, **C14-H**), 1.26 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.1 (**C2**), 187.0 (**C5**), 180.1 (**C12**), 158.6 (**C1**), 148.7 (**C4**), 129.8 (**C6**), 104.6 (**C3**), 66.7 (**C11**), 52.2

(**C15**), 36.0 (**C7**), 33.0 (**C13**), 29.9 (**C8**, **C9**, **C10**), 24.0 (**C14**); IR (KBr, cm^{-1}**):** 3377.2, 2962.2, 1623.5, 1581.5, 1424.5, 1146.2; CIMS (MeOH): 278.13828 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 276 (4.62), 498 (4.11).

4.1.1.16. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-methionine (**2g**)

Obtained by the same procedure as **2a**, using 130 mg *tert*-butylquinone and 118 mg L-methionine, but the reaction time was shortened to 2 h and extraction was done with chloroform. **2g**: red crystals; 43 mg (yield 17 %). Mp: 137–139 ºC (dec.); $[\alpha]_D^{20}$ = –24 (*c* 0.125, CH_3OH); ^1H NMR (200 MHz, CD_3OD): δ 6.42 (d, J= 2.2 Hz, 1H, **C6-H**), 5.44 (d, J= 2.2 Hz, 1H, **C4-H**), 3.88 (dd, J= 6.2 Hz, J= 11.8 Hz, 1H, **C11-H**), 2.52 (m, 2H, **C14-H**), 2.15-2.01 (m, 2H, **C13-H**), 1.98 (s, 3H, **C15-H**), 1.27 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.8 (**C5**), 184.0 (**C2**), 177.0 (**C12**), 153.2 (**C1**), 149.2 (**C3**), 135.8 (**C6**), 97.2 (**C4**), 58.0 (**C11**), 35.7 (**C7**), 32.5 (**C13**), 31.2 (**C14**), 29.4 (**C8**, **C9**, **C10**), 15.3 (**C15**); IR (KBr, cm^{-1}**):** 3344.6, 2961.2, 1671.4, 1630.4, 1580.6, 1488.2, 1423.6, 1339.5, 1143.7; CIMS (MeOH): 312.12628 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.77), 486 (4.31).

4.1.1.17. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-phenylalanine (**2h**)

Obtained by the same procedure as **2a**, using 230 mg *tert*-butylquinone and 232 mg L-phenilalanine. The reaction time was prolonged to 3 h. The product was rechromatographed by preparative TLC using toluene/ethyl acetate (9:1) as the eluent. **2h**: red crystals (186 mg, yield 41 %). Mp: 143–146 ºC; $[\alpha]_D^{20}$ = 16 (*c* 0.250, CH_3OH); ^1H NMR (200 MHz, CD_3OD): δ 7.22-7.14 (m, 5H, **C15**, **C16**, **C17**, **C18**, **C19**), 6.35 (d, J= 2.5 Hz, 1H, **C6-H**), 5.26 (d, J= 2.5 Hz, 1H, **C4-H**), 3.99 (dd, J=5.0 Hz, J= 7.4 Hz, 1H, **C11-H**), 3.23 (m, 1H, **C13-H_a**), 3.05 (dd, J= 7.2 Hz, J= 13.4 Hz, 1H, **C13-H_b**), 1.22 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.8 (**C5**), 183.8 (**C2**), 177.0 (**C12**), 153.0 (**C1**), 149.0 (**C3**), 138.8 (**C14**), 135.7 (**C6**), 130.5 (**C15**, **C19**), 129.4 (**C16**, **C18**), 127.7 (**C17**), 97.2 (**C4**), 60.1 (**C11**), 38.8 (**C13**), 35.6 (**C7**), 29.4 (**C8**, **C9**, **C10**); IR (KBr, cm^{-1}**):** 3353.7, 3060.6, 3027.9, 2960.2, 2870.3, 1672.5, 1627.9, 1573.3, 1502.0, 1449.9, 1400.6, 1365.3, 1340.8, 1256.6; CIMS (MeOH): 328.15398 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 274 (4.71), 488 (4.29).

4.1.1.18. *N*-(5-*tert*-Butyl-3,6-dioxo-1,4-cyclohexadien-1-yl)-L-tryptophan (**2i**)

Obtained by the same procedure as **2a**, but the reaction time was shortened to 1 h and the product was purified by preparative TLC using toluene/ethyl acetate (9:1) as the eluent. Starting from 100 mg *tert*-butylquinone and 125 mg L-tryptophane, **2i** was obtained as red crystals (42 mg, yield 18 %). Mp: 156–159 ºC (dec.); $[\alpha]_D^{20}$ = –24 (*c* 0.125, CH_3OH); ^{1}H NMR (200 MHz, CD_3OD): δ 7.56 (d, J= 8.0 Hz, 1H, **C12-H**), 7.30 (d, J= 8.0 Hz, 1H, **C17-H**), 7.13 (s, 1H, **C15-H**), 7.08-6.90 (m, 2H, **C18-H**, **C19-H**), 6.34 (d, J= 2.0 Hz, 1H, **C6-H**), 5.31 (poorly resolved d, 1H, **C4-H**), 4.11 (poorly resolved dd, 1H, **C11-H**), 3.49 (dd, J= 4.2 Hz, J= 14.4 Hz, 1H, **C13-H_a**), 3.27 (dd, J= 7.2 Hz, J= 14.4 Hz, 1H, **C13-H_b**), 1.18 (s, 9H, **C8-H, C9-H, C10-H**); ^{13}C NMR (50 MHz, CD_3OD): δ 187.7 (**C5**), 183.6 (**C2**), 177.6 (**C12**), 152.8 (**C1**), 149.1 (**C3**), 138.0 (**C16**), 135.7 (**C6**), 129.1 (**C21**), 124.5 (**C15**), 122.2 (**C20**), 119.7 (**C19**), 119.4 (**C18**), 112.2 (**C17**), 111.4 (**C14**), 96.9 (**C4**), 59.8 (**C11**), 35.5 (**C7**), 29.3 (**C8**, **C9**, **C10**), 28.8 (**C13**); IR (KBr, cm^{-1}**):** 3349.4, 3001.7, 2960.7, 2871.7, 1671.5, 1630.7, 1574.9, 1501.2, 1421.7, 1363.3, 1341.5, 1151.4; CIMS (MeOH): 367.16471 $[M+1]^+$; UV/Vis (MeOH): λ_{max} (log ε) 278 (4.90), 496 (4.27).

4.2. Pharmacology

4.2.1. Antiproliferative activity

4.2.1.1. Compounds and solutions

Stock solutions of investigated complexes, were prepared in DMSO at concentrations of 10 mM and afterwards they were diluted with complete nutrient medium (RPMI-1640 without phenol red) supplemented with 3 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10 % heat inactivated fetal bovine serum (FBS), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) adjusted to pH 7.2 by bicarbonate solution. RPMI-1640, FBS, Hepes and L-glutamine were products of Sigma Chemical Co., St. Louis, MO. The MTT (2-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl-2*H*-tetrazolium bromide) was dissolved (5 mg/ml) in phosphate buffer saline pH 7.2 and filtered (0.22 μm) before use. The RPMI 1640 cell culture medium, fetal bovine serum (FBS), and MTT, were purchased from Sigma Chemical Company, USA.

4.2.1.2. Cell lines

Cervix adenocarcinoma cell line (HeLa), human melanoma (Fem-X), human chronic myelogenous leukemia (K562) cells, human breast cancer (MDA-MB-453), non-small cell lung carcinoma (A549) and a non-cancerous cell line, MRC-5 (human embryonic lung fibroblast) were grown in RPMI-1640 medium (Sigma). Media were supplemented with 10 % fetal bovine serum, L-glutamine, and penicillin—streptomycin (Sigma).

4.2.1.3. Treatment of cell lines

Stock solutions (10 mM) of compounds, made in dimethyl sulfoxide (DMSO), were dissolved in corresponding medium to the required working concentrations. Neoplastic cells HeLa (2000 cells per well), Fem-X (5000 cells per well), K562 (5000 cells per well), MDA-MB-453 (3000 cells per well), A549 (5000 cells per well), and non-cancerous MRC-5 (5000 cells per well) were seeded into wells of a 96-well flat-bottomed microtitre plates, and 24 h later, after the cell adherence, five different, double diluted, concentrations of investigated compounds were added to the wells. Exceptionally, compounds were applied to the suspension of leukemia K562 cells (5000 cells per well), 2 hours after the cell seeding. Final concentrations applied to target cells were 200, 100, 50, 25, and 12.5 μM, except to the control wells where only nutrient medium was added to the cells. Nutrient medium was RPMI 1640 medium, supplemented with L-glutamine (3 mM), streptomycin (100 μg/ml), and penicillin (100 IU/ml), 10 % heat inactivated (56 ˚C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 h.

4.2.1.4. Determination of cell survival

The effect of the prepared compounds on cancer cell survival was determined by the microculture tetrazolium test (MTT) according to Mosmann [**47**] with modification by Ohno and Abe [**48**] 72 h after addition of the compounds, as described earlier. Briefly, 20 mL of MTT solution (5 mg/mL phosphate-buffered saline) was added to each well. Samples were incubated for a further 4 h at 37 ºC in a humidified atmosphere of 95 % air/5 % CO_2 (v/v). Then, 100 mL of 100 g/L sodium dodecyl sulfate were added to the extract, resulting in formation of insoluble formazan by conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was read in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm. The absorbance (*A*) at 570 nm was

measured 24 h later. To determine cell survival (%), *A* of the sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the control optical density (*A* of control cells grown only in nutrient medium) and multiplied by 100. It was implied that *A* of the blank was always subtracted from *A* of the corresponding sample with target cells. IC_{50} was defined as the concentration of an agent inhibiting cell survival by 50 % compared with a vehicle-treated control. As positive controls, cis-diamminedichloroplatinum (cis-DDP, cisplatin) was used. All experiments were done in triplicate.

4.2.1.5. Cell cycle analysis

Cervix adenocarcinoma cell line (HeLa) was seeded in six-well plates (3×10^5 cells/well), and after 24 h treated with investigated compounds, except control cells, and incubated at 37 °C for the next 24 hours. Concentrations used corresponded to IC_{50} values. After the incubation, the cells were collected by trypsinization, and fixed in ice-cold 70 % ethanol for 1h on ice, then at −20 °C for at least a week. After fixation, the cells were washed in PBS and pellets obtained by centrifugation were treated with RNase (100 μg/mL) at 37 °C for 30 min and then incubated with propidium iodide (PI) (40 μg/mL) for at least 30 min. DNA content and cell-cycle distribution were analyzed using a Becton Dickinson FACSCalibur flow cytometer. Flow cytometry analysis was performed using CellQuestR (Becton Dickinson, San Jose, CA, USA) software on a minimum of 10,000 cells per sample **[49]**.

4.2.1.6. Determination of target caspases

In order to examine the role of caspases involved in the apoptotic cell death induced by the investigated compounds, the percentages of HeLa cells pretreated with caspase inhibitors in sub-G1 phase were determined. HeLa cells were preincubated for 2 h with specific caspase inhibitors (final concentration e 40 mM): Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor) and Z-LEHD-FMK (caspase-9 inhibitor). Caspase inhibitors were purchased from R&D Systems (Minneapolis, USA). The tested compounds were applied to target HeLa cells at concentrations which corresponded to $2IC_{50}$ values obtained for 72 h. For each compound, one sample of HeLa cells was not treated with inhibitor and served as a referent sample. After 24 h of incubation, cells were harvested and fixed in 70 % ethanol on ice. Samples were stored at –20 ºC for one week before PI staining. Changes in the percentages of cells in sub-

G1 phase were determined using a FACSCalibur Flow Cytometer and analyzed using CellQuest Software.

4.2.2. Antibacterial activity

A microbroth double dilution test [**50**] was used to determine antibacterial activity of compounds against six Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Micrococcus flavus* (ATCC 10240), *Micrococcus luteus* (ATCC 4698), *Clostridium sporogenes* (ATCC 19404), *Kocuria rhizophila* (ATCC 9341), *Bacillus subtilis* (ATCC 6633), and four Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Proteus hauseri* (ATCC 13315), *Salmonella enterica* (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 9027). Sterile 96-well polystyrene microliter plates with well capacities of 300 µL were used, and 100 µL of fresh Mueller Hinton broth were added to each well of the plate. One hundred microliters of the compound stock solution (10 mg/mL) were added to the first well of the each row. Then, 100 µL of the solution were removed from the first well of the row to the following well of the same row and mixed with the broth previously added to it. This double dilution was done in every row of the plate. One row was used as a positive control containing a broad-spectrum antibiotic amikacin to determine the sensitivity of a bacterial strain. The other row contained the DMSO as a negative control. Afterwards, every well was inoculated with 10 µL (10^6 cells per mL) of bacterial cultures. The microtiter plate was incubated at 37 ºC for 24 h. The growth of bacteria was measured. The lowest concentration of tested compound which inhibited the bacterial growth was defined as MIC.

4.2.3. Antifungal activity

The tested fungi were *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763) and *Aspergillus brasiliensis* (ATCC 16404). Antifungal activities of the synthesized compounds were evaluated as previously described for antibacterial activity, by a microbroth double dilution assay. In this test the used broth was Sabouraud dextrose broth and each well was inoculated with 10 µL of fungal cultures (10^5 spores per mL). The microliter plate was incubated at 28 ºC for 48 h. The MIC was determined as the lowest concentration that resulted in inhibition of fungal growth.

4.2.4. The brine shrimp test

A small bag (18 g) of lyophilized eggs of brine shrimp *Artemia salina* was added to 0.5 L of tap water. A few grains of dried yeast were stirred in, and air was passed through the suspension where the temperature was maintained at 18–20 ºC under illumination for 48 h. Freshly hatched nauplii were used in further experiments [**51**].

All derivatives were dissolved in DMSO and various amounts (0.01–1 mg) were added to 950 µL of artificial seawater with 10–20 nauplii. The final concentration of DMSO was 5 %. After 24 h illumination at room temperature, the number of dead and surviving nauplii were counted and statistically analyzed. LC_{50} was defined as a concentration lethal to 50 % of the nauplii. All samples were done in triplicate.

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Figure captions

Fig. 1. Sesquiterpene quinones with an amino acid in side chain (nakijiquinones A–D; smenospongines B and C)

Fig. 2. Chemical structure of avarone and *tert*–butylquinone derivatives

Fig. 3. Tautomeric forms of avarone and t*ert*-butylquinone derivatives

Fig. 4. Cyclic voltammogram of compounds **1**, **1i**, **2** and **2i**

Fig. 5. Cell cycle distribution after 24 h continuous action of investigated compounds. Concentration corresponded to IC_{50}.

Fig. 6. Cell cycle distribution after 24 h continuous action of investigated compounds. Concentration corresponded to $2IC_{50}$.

Fig. 7. Influence of compounds **1c (A)** and **1e (B)** alone or in combination with inhibitors of caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) or caspase-9 (Z-LEHD-FMK) on the fraction of HeLa cells in sub-G1 phase.

Graphical abstract