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# New Chlorin–Terpene Conjugates: Synthesis, Photoinduced and Dark Cytotoxicity

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*Based on methylpheophorbide a, the number of chlorin-terpene conjugates containing myrtenyl, camphenyl, cyclobutane and cyclopropane fragments was obtained for the first time with 52–64% yields. Dark and photoinduced cytotoxic activity of the new conjugates was analyzed in comparison with methylpheophorbide a and terpenophenolic chlorin analogue in which terpene fragment was substituted by methyl group. The new compounds were also compared with Photolon, which is currently used in clinical practice. The results show that acetylated aminochlorin, and its conjugates with cis-pinonic and cis-pinononic acids and with 2,2-dimethyl-3-(2-oxopropyl)cyclopropyl acetic acid were characterized by enhanced dark cytotoxicity in comparison with methylpheophorbide a. At the same time, conjugation of aminochlorin with myrtenic or 3,3-dimethylbicyclo[2.2.1]heptane-2-carboxylic acids did not lead to the increase of dark cytotoxicity. The concentrations, at which chlorin-myrtenic (**3**) and chlorin-camphenylanic (**4**) conjugates exhibit photoinduced cytotoxicity, differ by more than two orders of magnitude from the concentrations at which these compounds show dark cytotoxicity. In comparison, the difference between the active concentrations under the light and in-the-dark for Photolon is approximately one order of magnitude. This allows to suggest a high potential of the new compounds **3** and **4** for further in vitro and in vivo studies to eventually improve the efficiency and safety of photodynamic therapy of cancer.*

**Keywords:** Chlorin $e_6$, terpene acids, chlorin-terpene conjugates, dicyclohexylcarbodiimide (DCC), photosensitizers.

# Новые хлорин–терпеновые конъюгаты: синтез, фотоиндуцированная и темновая цитотоксичность

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*На основе метилфеофорбида а впервые получен ряд терпен-хлориновых конъюгатов, содержащих миртенильный, камфенильный, циклобутановый и циклопропановый фрагменты с выходами 52–64 %. Оценена цитотоксичнсоть новых соединений в темноте и при активации красным светом в сравнении с метилфеофорбидом а, с аналогом терпен-хлориновых конъюгатов с метильной группой вместо терпенового фрагмента и используемым в клинической практике Фотолоном. Показано, что ацетилированный аминохлорин, а также конъюгаты аминохлорина с цис-пиноновой, цис-пинононовой и 2,2-диметил-3-(2-оксопропил)циклопропилуксусной кислотами характеризуются более высокой темновой цитотоксичностью, чем метилфеофорбид а. В то же время, конъюгирование аминохлорина с миртеновой и камфенилановой кислотами не приводит к увеличению темновой цитотоксичности относительно метилфеофорбида а. Концентрации, при которых проявляется*

*фотоиндуцированная цитотоксичность конъюгатов с фрагментами миртеновой (**3**) и камфенилановой (**4**) кислот в системе in vitro, отличаются на более чем два порядка от темновой, в то время как для используемого в клинической практике Фотолона эффективная концентрация действующего вещества на свету отличается от эффективной концентрации в темноте только на один порядок. Это позволяет предположить высокий потенциал новых соединений **3** и **4** для дальнейших исследований в системах in vitro и in vivo на пути повышения эффективности и безопасности фотодинамической терапии онкологических заболеваний.*

**Ключевые слова:** Хлорин $e_6$, терпеновые кислоты, хлорин-терпеновые конъюгаты, дициклогексилкарбодиимид, фотосенсибилизаторы.

## Introduction

Porphyrin cycle is an interesting object from the viewpoint of classical organic chemistry because it has a high potential to modify the structure. Besides that, it is highly prospective for development of bioactive compounds, since a number of chlorophyll derivatives are already used for medical purposes.[1] Primarily, different porphyrins are used to diagnose cancer because of their ability to accumulate in cancerous tissues to a greater extent than in healthy tissues.[2,3] Porphyrins are also used as photosensitizers for photodynamic cancer therapy because they show different cytotoxic activity under the light and in the dark.[4-6] Moreover, the new methods of target delivery of different photosensitizers, including porphyrin, to cancerous tissues are actively developed. Some of them use nanoparticles to do it.[7] Modification of the natural porphyrins by introduction of the terpene fragment on the periphery of the macrocycle can be very prospective because terpene derivatives express different bioactivity often based on different interactions with biomembranes. For example, the compounds that contain cyclobutane and cyclopropane rings are anti-viral and anti-germ compounds, neurotics and analgetics. In the chemical structure of these compounds there is a fragment of cis-pinonic acid which is easily derived from verbenone.[11-13] It can be assumed that the addition of the terpene moiety can improve the interaction with biological membranes compounds, enhancing the photosensitizing effect of the compound as a whole.

In this work we have synthesized chlorins which have macrocyclic fragments of terpene acids on their periphery. Moreover, we have analyzed dark and photoinduced cytotoxicity of these conjugates.

## Experimental

The conjugation of the chlorin macrocycle with terpene acids was done by synthesizing an amidic bond by the interaction of aminochlorin (**2**) with activated terpene acids. To the solution of terpene acid in 10 ml of the mixture of methylene chloride:pyridine (1:1) at 0 °C an equimolar amount of dicyclohexylcarbodiimide (DCC) (in mmol) was added. The mixture was shaken at 0 °C for 30 mins. Next, the amount of aminochlorin (**2**), equimolar to terpene acid, was added to the mixture. The reactive mixture was shaken at room temperature for 24 hours. Next, the reactive mixture was diluted with 70 ml of methylene chloride and pyridine was washed with 5 % hydrochloric acid, then with water. After that, the mixture was dried over dehydrated sodium sulfate and evaporated at low pressure. Evaporated precipitate was chromatographed on silica gel (eluent: tetrachloromethane – acetone, 3:1).

Acetylation of aminochlorin (compound **8**) was made using the method described earlier.[14]

$^1$H and $^{13}$C NMR spectra of the synthesized compounds were recorded on Bruker AVANCE-II-300 (working frequency 300 MHz and 75 MHz for NMR $^1$H and $^{13}$C respectively) using standard impulse Bruker software for one and two-dimensional experiments. Infrared spectra were measured in KBr tablets on the “IR Prestige 21” device (Shimadzu). Mass spectra were recorded on the “ThermoFinnigan LCQ Fleet” device. UV-Vis spectra were recorded on a spectrometer UV-1700 (Shimadzu) with the wavelength range of 200–1100 nm. The samples were analyzed in quartz cuvettes (10 mm thick). Chloroform was used as control. The reaction was controlled using TLC method on Sorbfil slides. Extraction of the reaction products was done using column chromatography on silica gel Alfa Aesar 70–230 mesh. Chemically clean pyridine for the reaction was dried in advance over the granules of KOH, then it was distilled over BaO. Chemically clean dichloromethane for the reaction was distilled over $P_2O_5$. The solvents for column chromatography, chemically clean tetrachloromethane and clean for analysis acetone, were used without additional purification.

*Compound **3**.* Yield 59 %, 108 mg, conversion 100 %. IR (KBr) ν cm$^{-1}$: 1735.93 (ν C=O, ester); 1602.85 (“chlorin band”); 1649.14 (“amide-I”); 1519.91 (“amide-II”). $^1$H NMR ($CDCl_3$, 300 MHz) δ ppm: the signals of protons of porphyrin fragment: 9.66 (s, 1H, 10); 9.50 (s, 1H, 5); 8.86 (s, 1H, 20); 7.89 (dd, 1H, 17.7 and 11.4 Hz, 3(1)-H); 7.06 (br, 1H, 13(2)-NH); 6.82 (br, 1H, 13(3)-NH); 6.15 (d, 1H, 17.7 Hz, 3(2trans); 5.99 (d, 1H, 11.4 Hz, 3(2cis); 5.36 (d, 1H, 19 Hz, 15(1`)), 5.15 (d, 1H, 19 Hz, 15(1``)); 4.53 (q, 1H, 7.1 Hz, 18); 4.42 (d, 1H, 9.1 Hz, 17); 3.75 (q, 2H, 7.5 Hz, 8(1)); 3.70 (s, 3H, 15(3)); 3.67 (s, 3H, 17(4)); 3.43 (s, 3H, 2(1)); 3.29 (s, 3H, 12(1)); 3.17-3.39 (m, 4H, 13(2), 13(3)); 3.12 (s, 3H, 7(1)); 2.61 (m, 1H, 17(2`), 2.25-2.35 (m, 2H, 17(2``), 17(1`)); 1.81 (m, 1H, 17(1”)), 1.78 (d, 3H, 7.1 Hz, 18(1)); 1.71 (t, 3H, 7.5 Hz, 8(2)); -1.67 (br, 1H, I-NH), -1.84 (br, 1H, III-NH). The signals of protons of terpene fragment: 0.81 (s, 3H, 8′-$CH_3$), 1.13 (m, 1H, 9 Hz, 7′-CH), 1.31 (s, 3H, 9′-$CH_3$); 2.09 (m, 1H, 5′-H), 2.30-2.40 (m, 2H, 4′-$CH_2$), 2.46 (m, 1H, 7′-CH), 2.71 (m, 1H, 1′-CH), 6.39 (m, 1H, 3′-CH). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ ppm: the signals of carbons of porphyrin fragment: 173.6 (17(3)), 173.5 (15(2)), 170.5 (13(1)), 169.2 (19), 167.0 (16), 144.6 (8), 139.1 (1), 136.0 (7), 135.0 (14), 134.9 (4), 134.8 (11), 134.7 (3), 130.3 (2), 129.8 (12), 129.2 (3(1)), 127.8 (13), 121.7 (3(2)), 102.4 (15), 101.3 (10), 98.7 (5), 93.9 (20), 53.1 (17), 52.2 (17(4)), 51.6 (15(3)), 49.3 (18), 40.6 (13(2)), 40.2 (13(1)), 37.7 (15(1)), 31.1 (17(2)), 29.7 (17(1)), 23.1 (18(1)), 19.7 (8(1)), 17.6 (8(2)), 12.1 (2(1)), 11.7 (12(1)), 11.2 (7(1)). The signals of carbons of terpene fragment: 167.7 (10`), 143.2 (2`), 129.5 (3`), 41.6 and 40.4 (1` and 5`), 37.7 (6`), 31.8 (4`), 31.4 (7`), 26.0 (9`), 21.0 (8`). Mass spectrum, *m/z*: 815.37 $[M+H]^+$.

*Compound **4**.* Yield 52 %, 95 mg, conversion 100 %. IR (KBr) ν cm$^{-1}$: 1735.93 (ν C=O, ester); 1600.92 (“chlorin band”);

1649.14 ("amide-I"); 1517.98 ("amide-II"). $^1$H NMR (DMSO-$d_6$, 300 MHz) δ ppm: the signals of protons of porphyrin fragment: 9.81 (s, 1H, 10-H); 9.74 (s, 1H, 5-H); 9.13 (s, 1H, 20-H); 9.17 (br.m, 1H, 13(1)-NH (amide)); 8.29 (dd, 1H, 18.0 and 11.7 Hz, 3(1)-H); 7.73 (br.m, 1H, 13(3)-NH (amide)); 6.43 (d, 1H, 18.0 Hz, 3(2)-H (trans)); 6.16 (d, 1H, 11.7 Hz, 3(2)-H (cis)); 15(1)-$CH_2$: 5.55 (d, 1H, 19 Hz), 5.37 (d, 1H, 19 Hz); 4.65 (q, 1H, 7.0 Hz, 18-H); 4.45 (d, 1H, 9.3 Hz, 17-H); 3.83 (q, 2H, 8(1)-$CH_2$, 7.3 Hz); 3.76-3.64 (m, 4H, 13(2)-$CH_2$, 13(3)-$CH_2$); 3.72 (s, 3H, 15(3)-$CH_3$); 3.60 (s, 3H, 17(4)-$CH_3$); 3.54 (s, 3H, 12(1)-$CH_3$); 3.52 (s, 3H, 2(1)-$CH_3$); 3.31 (s, 3H, 7(1)-$CH_3$); 17(1)-$CH_2$, 17(2)-$CH_2$: 2.79-2.65 (m, 2H), 2.45-2.25 (m, 2H); 1.74-1.62 (m, 6H, 18(1)-$CH_3$, 8(2)-$CH_3$); -1.77 (br.s, 1H, I-NH), -2.04 (br.s, 1H, III-NH). The signals of protons of terpene fragment: 0.96 (s, 3H, 9′-$CH_3$), 1.07 (s, 3H, 10′-$CH_3$), 1.18 (m, 1H, 9 Hz, 7′endo-CH), 1.79 (m, 1H, 4′-CH), 1.79 (m, 1H, 2′-CH), 2.05-2.12 (m, 2H, 6′-$CH_2$), 2.23-2.31 (m, 2H, 5′-$CH_2$), 2.39 (m, 1H, 1′-CH), 2.42 (m, 1H, 7′exo-CH). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ ppm: the signals of carbons of porphyrin fragment: 174.3 and 173.5 (15(2) and 17(3)), 173.4 (both amide), 170.1 (13(1)), 169.6 (16), 167.5 (19), 144.3(8), 139.4 (1), 135.9 (7), 134.8 (11), 130.4 (2), 130.0 (13), 129.1 (3(1)), 121.9 (3(2)), 102.7 (15), 101.2 (10), 98.7 (5), 94.2 (20), 56.8 (2`), 53.2 (17), 52.2 (17(4)), 51.7 (15(3)), 49.3 (18), 49.1 (4`), 41.7 (1`), 40.6 and 39.7 (13(2) and 13(3)), 38.1 (15(1)), 38.0, 24.5 and 21.5 (5`, 6` and 7`), 37.8 (3`), 32.5 (9`), 31.2 (17(2)), 29.7 (17(1)), 23.1 (18(1)), 22.7 (8`), 19.7 (8(1)), 17.6 (8(2)), 12.1 (2(1)), 11.9 (12(1)), 11.2 (7(1)). The signals of carbons of terpene fragment: 180.3 (10`), 56.4 (2`), 49.0 (4`), 40.8 (1`), 38.5 (3`), 37.6 (7`), 31.9 (8`), 24.6 (6`), 22.8 (9`), 21.4 (5`). Mass spectrum, *m/z*: 817.40 $[M+H]^+$.

*Compound* ***5***. Yield 60 %, 110 mg, conversion 100 %. IR (KBr) ν cm$^{-1}$: 1735.93 (ν C=O, ester); 1600.92 ("chlorin band"); 1654.92 ("amide-I"); 1519.91 ("amide-II"). $^1$H NMR ($CDCl_3$, 300 MHz) δ ppm: the signals of protons of porphyrin fragment: 9.64 (s, 1H, 10-H); 9.53 (s, 1H, 5-H); 8.85 (s, 1H, 20-H); 7.94 (dd, 1H, 17.9 and 11.6 Hz, 3(1)-H); 6.79 (br.m, 1H, 13(1)-NH (amide)); 6.53 (br.m, 1H, 13(3)-NH (amide)); 6.21 (d, 1H, 18.0 Hz, 3(2)-H (trans)); 6.02 (dd, 1H, 11.6 and 1.1 Hz, 3(2)-H (cis)); 15(1)-$CH_2$: 5.41 (d, 1H, 18.9 Hz), 5.17 (d, 1H, 18.9 Hz); 4.53 (q, 1H, 7.1 Hz, 18-H); 4.42 (d, 1H, 8.7 Hz, 17-H); 3.81-3.67 (m, 2H, 8(1)-$CH_2$); 3.35-3.20 (m, 4H, 13(2)-$CH_2$, 13(3)-$CH_2$); 3.70 (s, 3H, 17(4)-$CH_3$); 3.66 (s, 3H, 15(3)-$CH_3$); 3.44 (s, 3H, 2(1)-$CH_3$); 3.30 (s, 3H, 2(12)-$CH_3$); 3.16 (s, 3H, 7(1)-$CH_3$); 17(1)-$CH_2$: (m, 1.85 and 2.30), 17(2)-$CH_2$: (m, 2.23 and 2.65), 1.77 (d, 3H, 7.2 Hz, 18(1)-$CH_3$); 1.72 (t, 3H, 7.5 Hz, 8(2)-$CH_3$); –1.60 (br, 1H, I-NH), –1.82 (br, 1H, III-NH). The signals of protons of terpene fragment: 2.50 and 1.70 (m, by 1H, 4`-H), 2.50 (m, 1H, 1′-H); 2.32 (m, 1H, 3′-H); 1.89 (s, 3H, 6′-$CH_3$); 1.21 (s, 3H, 9′-$CH_3$), 0.84 (s, 3H, 8′-$CH_3$). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ ppm: the signals of carbons of porphyrin fragment: 173.7 (17(3)), 173.5 (15(2)), 170.3 (13(1)), 169.1 (19), 167.0 (16), 144.7 (8), 139.0 (1), 136.1 (7), 134.9, 134.8, 134.6, 130.3 (2), 129.7 (12), 129.2 (3(1)), 127.7 (13), 121.7 (3(2)), 102.3 (15), 101.3 (10), 98.8 (5), 93.8 (20), 53.1 (17), 52.2 (17(4)), 51.7 (15(3)), 49.2 (18), 40.4 and 39.9 (13(2) and 13(3)), 37.7 (15(1)), 31.1 (17(2)), 29.7 (17(1)), 23.1 (18(1)), 19.6 (8(1)), 17.7 (8(2)), 12.1 (2(1)), 11.7 (12(1)), 11.2 (7(1)). The signals of carbons of terpene fragment: 207.2 (C=O), 171.8 (amide), 52.8 (1`), 46.2 (3`), 44.5 (2`), 30.4 (9`), 29.8 (6`), 19.0 (4`), 17.7 (8`). Mass spectrum, *m/z*: 819.40 $[M+H]^+$.

*Compound* ***6***. Yield 64 %, 120 mg, conversion 100 %. IR (KBr) ν cm$^{-1}$: 1735.93 (ν C=O, ester); 1600.92 ("chlorin band"); 1654.92 ("amide-I"); 1525.69 ("amide-II"). $^1$H NMR ($CDCl_3$, 300 MHz) δ ppm: the signals of protons of porphyrin fragment: 9.67 (s, 1H, 10-H); 9.56 (s, 1H, 5-H); 8.85 (s, 1H, 20-H); 7.98 (dd, 1H, 18 and 11.5 Hz, 3(1)-H); 6.99 (br, 1H, 13(1)-NH (amide)); 6.31 (br, 1H, 13(3)-NH (amide)); 6.24 (d, 1H, 18.0 Hz, 3(2)-H (trans)); 6.06 (d, 1H, 11.5, 3(2)-H (cis)); 15(1)-$CH_2$: 5.42 (d, 1H, 19 Hz), 5.19 (d, 1H, 19 Hz); 4.52 (q, 1H, 6.8 Hz, 18-H); 4.42 (d, 1H, 8.8 Hz, 17-H); 3.78 (q, 2H, 7.5 Hz, 8(1)-$CH_2$); 3.18-3.01 (m, 4H, 13(2)-$CH_2$, 13(3)-$CH_2$); 3.71 (s, 3H, 17(4)-$CH_3$); 3.67 (s, 3H, 15(3)-$CH_3$); 3.46 (s, 3H, 2(1)-$CH_3$); 3.34 (s, 3H, 12(1)-$CH_3$); 3.21 (s, 3H, 7(1)-$CH_3$); 2.63-2.25 (m, 4H, 17(1)-$CH_2$, 17(2)-$CH_2$); 1.80 (d, 3H, 6.9 Hz, 18(1)-$CH_3$); 1.75 (t, 3H, 6.9 Hz, 8(2)-$CH_3$); -1.64 (br, 1H, I-NH), -1.83 (br, 1H, III-NH). The signals of protons of terpene fragment: 2.58 (m, 1H, 3′-H); 2.30 (m, 1H, 1′-H); 1.85 (s, 3H, 6′-$CH_3$); 1.80 and 1.72 (m, 2H, 7′-$CH_2$); 1.90-1.72 (m, 2H, 4′-$CH_2$); 1.09 (s, 3H, 10′-$CH_3$); 0.66 (s, 3H, 9′-$CH_3$). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ ppm: the signals of carbons of porphyrin fragment: 173.6 and 173.5 (17(3) and 15(2)), 170.1 (13(1)), 169.3 (16), 167.2 (19), 144.7 (8), 139.2 (1), 136.1 (7), 135.1 (11), 135.0 (3), 134.9 (4), 134.8 (14), 130.4 (2), 129.7 (12), 129.2 (3(1)), 127.8 (13), 121.9 (3(2)), 102.4 (15), 101.3 (10), 98.7 (5), 94.0 (20), 53.1 (17), 52.2 (17(4)), 51.7 (15(3)), 49.3 (18), 40.5 (13(2)), 39.5 (13(1)), 37.8 (15(1)), 31.1 (17(2)), 29.7 (17(1)), 23.1 (18(1)), 19.7 (8(1)), 17.7 (8(2)), 12.1 (2(1)), 11.8 (12(1)), 11.2 (7(1)). The signals of carbons of terpene fragment: 207.6 (C=O), 172.6 (amide), 54.0 (3`), 43.2 (2`), 38.1 (1`), 36.7 (7`), 29.9 (6`), 29.9 (10`), 23.0 (4`), 17.2 (9`). Mass spectrum, *m/z*: 833.40 $[M+H]^+$.

*Compound* ***7***. Yield 52 %, 98 mg, conversion 100 %. IR (KBr) ν cm$^{-1}$: 1735.93 (ν C=O, ester); 1600.92 ("chlorin band"); 1653.00 ("amide-I"); 1527.62 ("amide-II"). $^1$H NMR ($CDCl_3$, 300 MHz) δ ppm: the signals of protons of porphyrin fragment: 9.70 (s, 1H, 10-H); 9.63 (s, 1H, 5-H); 8.85 (s, 1H, 20-H); 8.06 (dd, 1H, 18 and 11.5 Hz, 3(1)-H); 7.32 (br, 1H, 13(1)-NH (amide)); 6.91 (br, 1H, 13(3)-NH (amide)); 6.33 (d, 1H, 18.0 Hz, 3(2)-H (trans)); 6.13 (d, 1H, 11.5, 3(2)-H (cis)); 15(1)-$CH_2$: 5.58 (d, 1H, 18.7 Hz), 5.25 (d, 1H, 18.7 Hz); 4.52 (q, 1H, 7.0 Hz, 18-H); 4.41 (d, 1H, 9.0 Hz, 17-H); 3.87-3.77 (m, 2H, 8(1)-$CH_2$); 3.71-3.43 (m, 4H, 13(2)-$CH_2$, 13(3)-$CH_2$); 3.78 (s, 3H, 17(4)-$CH_3$); 3.67 (s, 3H, 15(3)-$CH_3$); 3.52 (s, 3H, 2(1)-$CH_3$); 3.50 (s, 3H, 12(1)-$CH_3$); 3.28 (s, 3H, 7(1)-$CH_3$); 2.67-1.90 (m, 4H, 17(1)-$CH_2$, 17(2)-$CH_2$); 1.76 (d, 3H, 7.0 Hz, 18(1)-$CH_3$); 1.74 (t, 3H, 7.8 Hz, 8(2)-$CH_3$); -1.53 (br, 1H, I-NH), –1.78 (br, 1H, III-NH). The signals of protons of terpene fragment: 2.31+2.08 (m, by 1H, 4′-$CH_2$); 2.23+1.88 (m, by 1H, 7′-$CH_2$); 1.52 (s, 3H, 6′-$CH_3$); 1.03 (s, 3H, 10′-$CH_3$); 0.79 (s, 3H, 9′-$CH_3$); 0.85 (m, 1H, 3′-H); 0.75 (m, 1H, 1′-H). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ ppm: the signals of carbons of porphyrin fragment: 173.5 and 173.3 (17(3) and 15(2)), 170.0 (13(1)), 168.8 (19), 166.8 (16), 154.2 (6), 149.1 (9), 144.8 (8), 138.9 (1), 136.1 (7), 135.1 (14), 134.9 (11), 134.8 (4), 134.6 (3), 130.2 (2), 129.8 (12), 129.4 (3(1)), 128.1 (13), 121.6 (3(2)), 102.3 (15), 101.4 (10), 98.9 (5), 93.7 (20), 53.1 (17), 52.2 (17(4)), 51.6 (15(3)), 49.2 (18), 40.5 (13(2)), 39.9 (13(3)), 37.8 (15(1)), 31.2 (17(2)), 29.7 (17(1)), 23.1 (18(1)), 19.7 (8(1)), 17.7 (8(2)), 12.1 (2(1)), 11.9 (12(1)), 11.3 (7(1)). The signals of carbons of terpene fragment: 210.0 (C=O), 174.0 (amide), 38.8 (4`), 31.9 (7`), 29.4 (6`), 28.4 (10`), 22.2 (3`), 21.4 (1`), 16.7 (2`), 15.1 (9`). Mass spectrum, *m/z*: 833.49 $[M+H]^+$.

The photoinduced and dark cytotoxicity of the new compounds were estimated using HeLa cells. The cells were cultivated without antibiotics in the growth media DMEM/F12 (PAALaboratoriesGmbH, Austria), containing 10 % of fetal calf serum (Thermo Scientific HyClone, UK) at 37 °C, 100 % humidity, 5 % $CO_2$.

The stock solutions of the tested compounds were prepared by dilution in DMSO (Amresco, USA) in different concentrations. One µl of the respective stock solution was put into 199 µl of growth media which contained 5000 cells in the sterile culture plate wells. The final concentrations were ranging from 0.01 to 100 µM. During the analysis of dark cytotoxicity, the cells were incubated with the tested compounds for 72 hours under the cultivation conditions in the dark. For analysis of the photoinduced toxicity after two hours of incubation the cells were exposed to red light (660 nm) for 20 minutes. The matrix of 96 light-emitting diode (60 mW each) was used as a source of light. Diodes were placed in the same way as the 96 wells of the culture plate and were connected in the 12 groups of 8 diodes and powered through the current stabilizers which support 15 mA. This way reached equal light emission of all the diodes (426 mlm through each well). Exposition to light was done through the bottom of the culture plate. The distance between the light source and the plate was 3 mm. After irradiation with light the cells with tested compounds were incubated further for 70 hours in the dark under the conditions mentioned earlier. Then,

the growth media was removed and the monolayered culture was washed with 200 μl of salt-phosphate buffer solution. Next, 100 μl of fluorescein diacetate solution (Sigma, USA) was added into the wells and the plates were left in the $CO_2$ incubator for 40 min. After that the measurements of fluorescence were recorded using liquid analyzer "Fluorat-02-Panorama" (LTD "Lumex", Russia) at the wavelength of 485 (excitation)/520 (registration) nm. The relative number of living cells was estimated using FMCA method as described in Lindhagen *et al.* (2008).[15] The experiments were repeated in 9–12 microcultures per each variant of experimental conditions. Average values were used in the report.

## Results and Discussion

Synthesis of aminochlorin (**2**) was done through opening of methylpheophorbide *a* (**1**) exocycle with ethylene diamine.[16] Chlorin terpene derivatives (**3**–**7**) were obtained by interaction of the activated carboxyl group of terpene acids with aminochlorin. The initial acids were: myrtenic,[17] 3,3-dimethylbicyclo[2.2.1]heptane-2-carboxylic,[18] cis-pinonic,[19] cis-pinononic,[19] and 2,2-dimethyl-3-(2-oxopropyl)cyclopropyl acetic acids[20] (Figure 1).

Activation of the carboxyl groups of the terpenic acids was done using DCC.[21] Formation of chlorin-terpene derivatives was conducted under mild conditions, that is why the reaction yield was 52–64 % with a 100 % conversion of the initial aminochlorin (**2**).

The structures of all the synthesized compounds were confirmed by electronic, IR and NMR spectroscopy and also by mass-spectrometry.

NMR spectra of chlorin conjugate **3** with myrtenic acid have the signals of porphyrinic and myrtenic fragments. In the NMR spectra for porphyrinic cycles there were observed only shifts of signals 13(2) and 13(3) into the weak field. For terpene substituent there were seen the shifts of signal of carbon atoms at the double bond between C2' and C3' in NMR $^{13}C$ spectrum, and shift of the signal of proton 3H' at the double bond in NMR $^{1}H$ spectrum. There is a broad singlet in the region of 6.40 ppm in the NMR $^{1}H$ spectrum of the chlorin-terpene derivative **3** in comparison with the spectra of the initial conjugates of aminochlorin **2** and terpenic acid. This wide singlet corresponds to the proton of amidic group in the position 13(3) which appears during the reaction. The evidence of covalent bond formation between chlorin and terpene fragments is the presence of H-C correlation in HMBC spectrum between the protons NH at the carbon 13(3) chlorin atom and the atom of carbon 10' of carbonyl group of terpene. In the mass-spectrum of the synthesized compound **3** there is a signal of protonated molecular ion $[M+H]^+$ what also confirms the structure of the reaction product. The structures of other chlorin-terpene conjugates **4**–**7** were confirmed by the same way.

For the synthesis of conjugate **4** the mixture of 3,3-dimethylbicyclo[2.2.1]heptane-2-carboxylic acid and its isomeric forms (endo- and exo-isomers at the position of carboxyl group). Only endo-isomer of 3,3-dimethylbicyclo[2.2.1]heptane-2-carboxylic acid has reacted. Most likely in the case of exo-isomer the activation did not happen because of sterical obstacles at the site of interaction

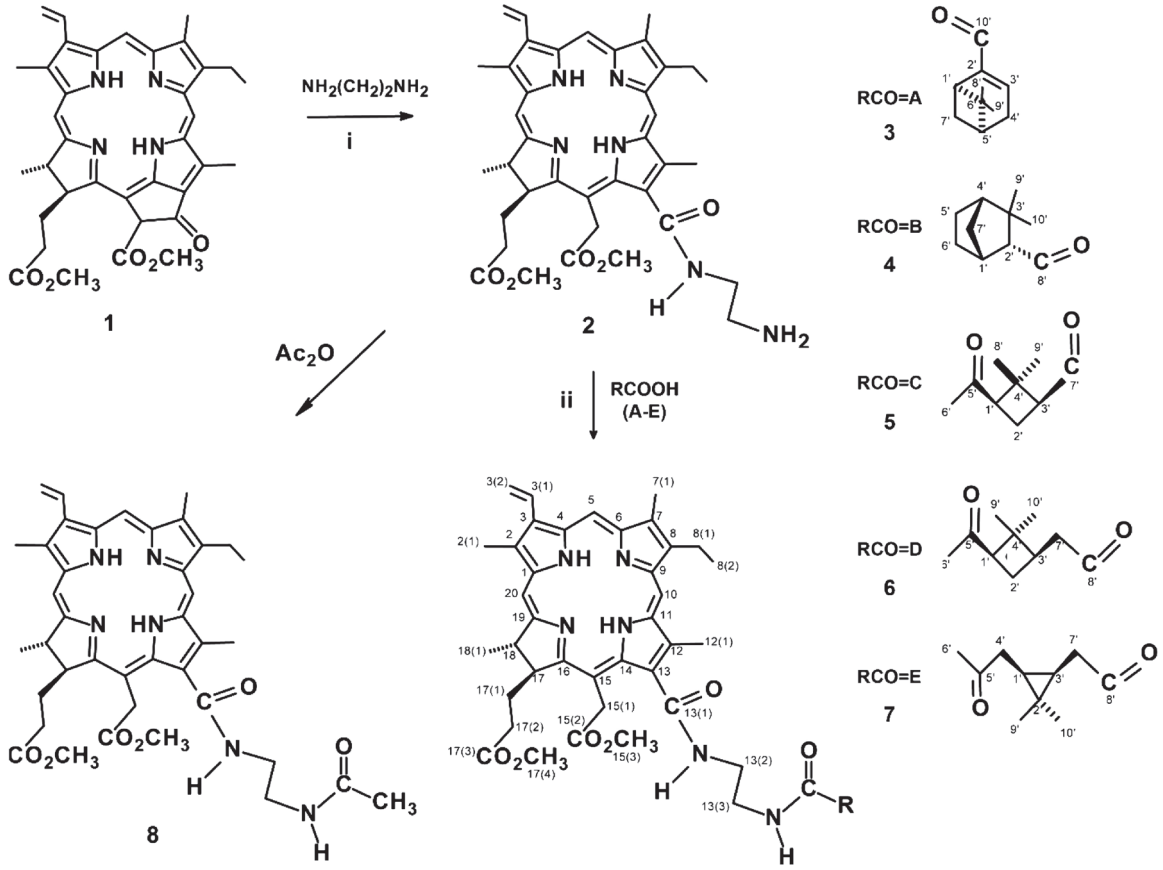


**Figure 1.** $CHCl_3$, ethylenediamine, 22 ºC, 3 h.; ii: $CH_2Cl_2$–pyridine, DCC, terpene acid, 22 °C, 24 h; iii: $Ac_2O$-pyridine, 22 °C, 1 h. Acid residues: A – myrtenic, B – camphenylanic, C – pinonic, D – pinononic, E – 2,2-dimethyl-3-(2-oxopropyl)cyclopropyl acetic.

with DCC. That is why the corresponding derivative was not formed.

Conjugation of aminochlorin with residues of pinonic acid, pinononic and 2,2-dimethyl-3-(2-oxopropyl) cyclopropyl acetic acids (compounds **5**, **6** and **7** respectively), as well as acetylation, both lead to an increase of dark cytotoxicity of the compounds in comparison with initial methylpheophorbide *a* and Photolon which is used in clinical practice. On the contrary, introduction of the mirtenic and camphelanic acid fragments (compounds **3** and **4**) does not lead to any increase of dark cytotoxicity. The values of cell survival under the maximum concentration (100 μM) of the tested compounds stay within the respective values for Photolon (in terms of the active compound) and methylpheophorbide *a* (Table 1).

Photoinduced activity of the compounds was studied after a 20 min exposure to red light (660 nm) at the beginning of cell incubation. Methylpheophorbide *a*, which was the least toxic in-the-dark, had the photoinduced cytotoxic activity one order of magnitude higher than that of chlorin $e_6$. The treatment with new conjugates **3** and **4** (at the concentrations which are two orders of magnitude lower than the photoactive concentration of chlorin $e_6$) leads to the almost complete termination of the cells after activation with red light (Figure 2).

## Conclusion

Significant photoinduced cytotoxicity of the new compounds **3** and **4** *in vitro* appears at the concentrations that are more than two orders of magnitude different from the dark one. In comparison, for chlorin $e_6$, which is used in clinic practice, under the same conditions effective photoinduced concentration is only one order of magnitude less than active concentration in dark. This allows to suggest a high potential

**Table 1.** Dark cytotoxic activity of the investigated compounds identified using FMCA method on the HeLa cells (expressed as a fraction of living cells in the treated microcultures in comparison with the untreated ones).

| Compound | Survival index, % | | | | |
|---|---|---|---|---|---|
| | 0.01 μM | 0.1 μM | 1 μM | 10 μM | 100 μM |
| Methylpheophorbide (**1**) | 89.01±13.53 | 89.03±14.38 | 81.57±11.66 | 72.84±8.73 | 40.60±4.39 |
| **3** | 87.93±2.18 | 77.35±2.32 | 75.23±2.53 | 62.36±1.05 | 2.23±0.20 |
| **4** | 96.51±2.28 | 79.01±2.53 | 82.82±3.62 | 69.52±3.61 | 17.62±2.54 |
| **5** | 92.44±3.00 | 81.67±3.50 | 83.49±3.39 | 0.77±0.05 | 0.62±0.03 |
| **6** | 99.32±1.76 | 91.94±2.69 | 91.60±2.46 | 6.17±0.40 | 1.46±0.18 |
| **7** | 106.41±4.28 | 85.88±3.04 | 91.96±1.58 | 0.75±0.02 | 0.81±0.01 |
| **8** | 88.76±2.24 | 85.17±2.94 | 81.34±3.56 | 0.41±0.05 | 0.37±0.02 |
| Photolon® * | 101.15±2.38 | 81.63±2.60 | 71.96±1.42 | 64.55±2.61 | 0.24±0.01 |

*the concentration of active compound (chlorin $e_6$) is shown.

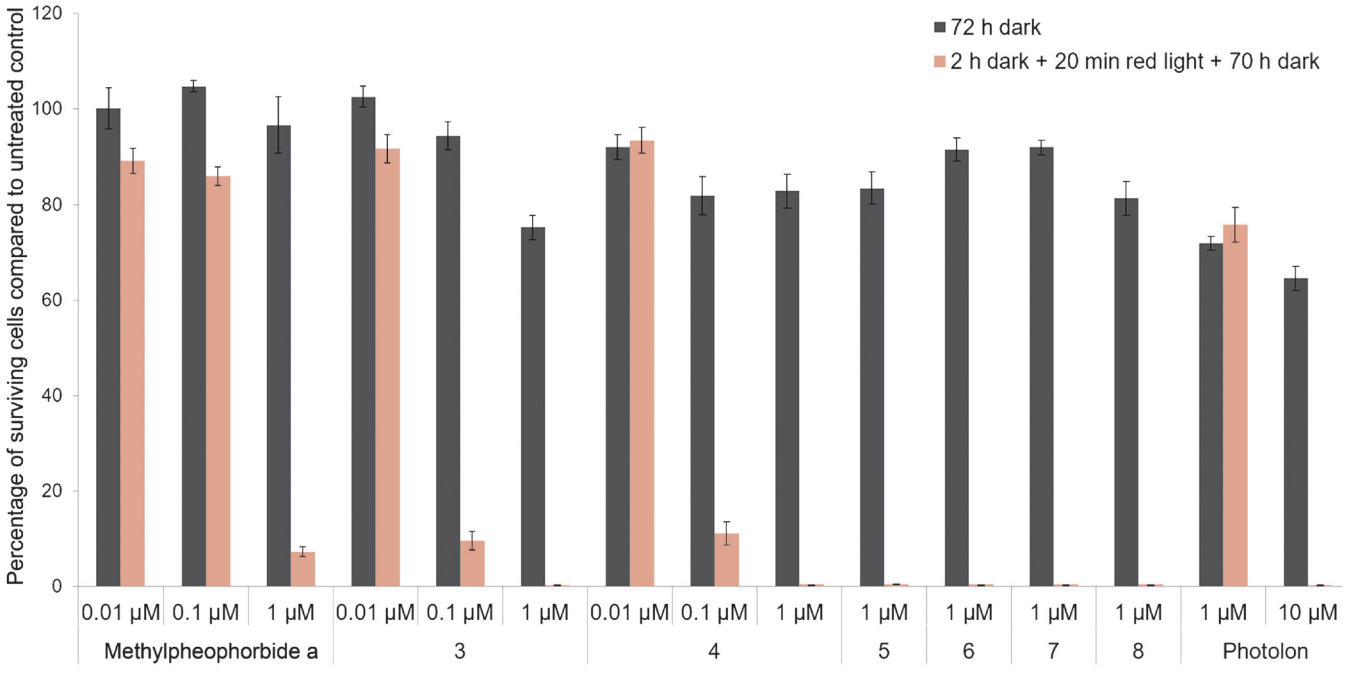


**Figure 2.** The survival of HeLa cells (estimated by FMCA) treated with the new compounds **3**–**8**, methylpheophorbide *a* (**1**) and a medical drug Photolon® (in terms of concentration of active substances – chlorin $e_6$) for 72 hours in-the-dark and with red light exposure step (660 nm) for 20 min.

of the new compounds **3** and **4** in development of more effective photodynamic oncotherapy.

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