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Synthesis and Properties of a Fluorescent-Labeled Triglyceride Derivative of the Antitumor Drug Merphalan

I. A. Boldyrev, I. V. Grechishnikova, Yu. B. Pavlova, and Jul. G. Molotkovsky[1]

Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, GSP Moscow, 117997 Russia

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Abstract—A new fluorescent probe, a 3-perylenoyl derivative of the lipophilized antitumor drug merphalan (sarcolysine), was synthesized. The probe is suitable for studying intracellular traffic and metabolism of merphalan and its derivatives. The perylenoyl fluorescence is partially quenched by the merphalan chromophore, which broadens the probe potentialities.

Key words: fluorescent probe, merphalan, 3-perylenoyl, synthesis

INTRODUCTION

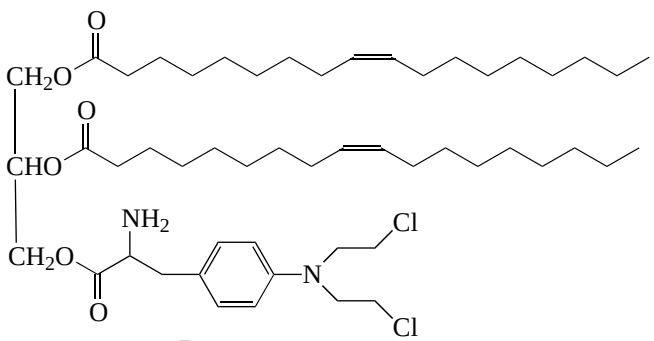
Liposomal preparations for the delivery of anticancer drugs are now actively studied. In recent years, they have also found a practical use, since, in the majority of cases, their cytotoxic efficiency increases and their systemic toxicity decreases (see, e.g., review [1]). When designing new formulations of anticancer drugs for such delivery, we have previously reported the synthesis of *rac*-1,2-dioleoyl-3-sarcolysylglycerol (**I**) [2], a lipophilic derivative of merphalan, (4-[bis(2-chloroethyl)amino]-*DL*-phenylalanine. This compound manifested a substantial cytotoxic activity in tumor cell cultures *in vitro* [3] and an appreciable antitumor activity *in vivo* [4, 5].

The knowledge of pathways and mechanisms of drug penetration into cells and tissues is of great importance for drug design. At present, fluorescence methods, in particular, the confocal fluorescence microscopy (CFM), are widely used for these studies (see, e.g., [6]). A prerequisite for using this method must be either an intrinsic fluorescence of a studied compound [7] or a fluorophore label in its molecule. Bearing in mind studying pharmacodynamics of (**I**) and similar compounds using CMF and other fluorescence techniques, we synthesized its fluorescent analogue, *rac*-1-(*Z*-9-octadecenyl)-2-sarcolysyl-3-[3-(3-perylenoyl)propionyl]-glycerol (**V**) (scheme). This bears a fluorescent perylenoyl residue, which proved to be a highly efficient probe in fluorescent microscopy and flow cytometry [8].

[1] Corresponding author; phone/fax: +7 (095) 330-6601; e-mail: jgmol@ibch.ru

RESULTS AND DISCUSSION

1-Octadecenylglycerol (selachyl alcohol) (**II**) was used as a starting compound in the synthesis. The hydrophobic chain in this compound is linked to the glycerol residue with ether instead of ester bond, which a little differentiates it from triglyceride (**I**) in structure. However, ether bond is very close to ester bond in its polarity and is more stable to the hydrolysis by intracellular catabolic enzymes. Due to this, our probe may live longer in the studied system. The acylation of selachyl alcohol with 3-(3-perylenoyl)propionic acid by carbodiimide method predominantly proceeded at position 3 of glycerol residue; (**III**) was isolated by column chromatography. Its acylation with *N*-Boc-merphalan by carbodiimide method resulted in (**IV**), which was deprotected to the desired probe (**V**) by treatment with trifluoroacetic acid. In addition to the synthetic scheme and chromatographic behavior, the structures



(**I**)

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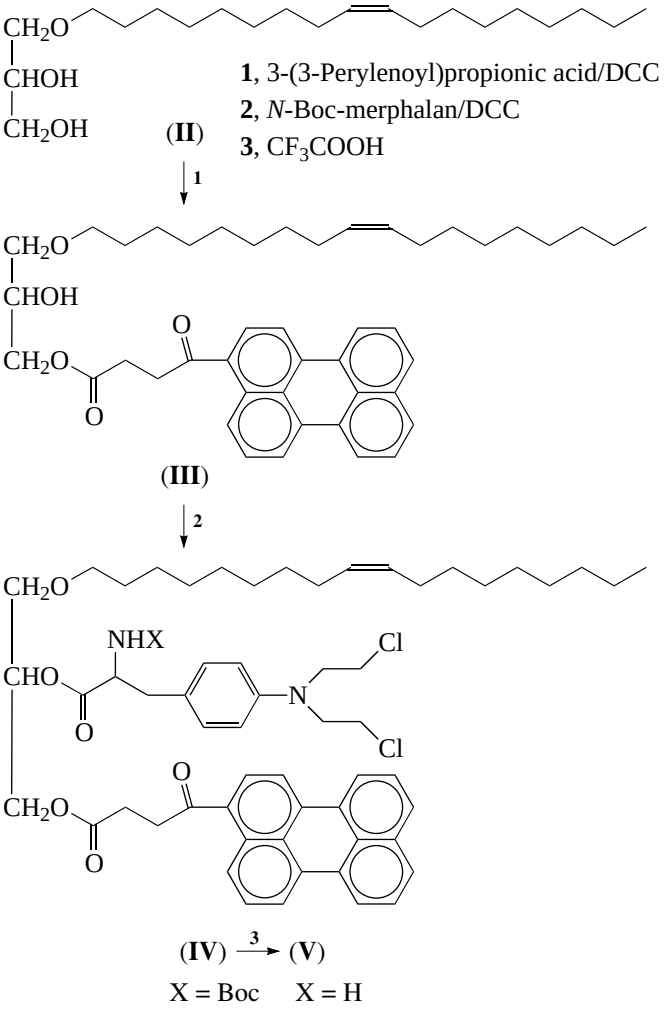

Scheme

of (**III**)–(**V**) were above all confirmed by ^{1}H NMR and mass spectra. Their fluorescence spectra were characteristic of perylenoyl compounds [9] and demonstrated λ_{ex} 450–452 nm and λ_{em} 526–527 nm in ethanol (see the Experimental section).

The fluorescent perylenoyl group in the probe (**V**) as well as in the synthon (**IV**) is sufficiently close to the *p*-bis(2-chloroethyl)aminophenyl group of merphalan, which could quench the fluorophore. We have not found in literature any data concerning the application of the merphalan residue as a fluorescence quencher. However, when planning the synthesis, we have presumed such a capability of this residue, which followed from the basic principles of this phenomenon, which are now well-known [10]. It turned out that the fluorescence of (**IV**) and (**V**) is indeed partially quenched in comparison with the emission of (**III**). Relative quantum yield, represented as a ratio of the fluorescence intensity at the emission maximum (in relative units) to the molar extinction coefficient at the absorption maximum [11] in the same sample, is 1.00 for (**III**), 0.59 for (**IV**), and 0.58 for (**V**) (in ethanol). In other words, the quantum yield of the perylenoyl fluorophore in both (**IV**) and (**V**) is almost two times lower than the normal value due to the quenching by the merphalan residue.

Obviously, the separation of these residues upon the cleavage of one of the ester bonds in the probe (**V**), for example, under the action of esterases will be accompanied by an increase in the fluorescence intensity. Such a methodological approach, when fluorophore and quencher are combined in the same molecule and their enzymatic separation enables studying the enzyme activity, is attracting more and more attention (see, e.g., [12]). In our case, this property of probe (**V**) widens its potentialities in studying the behavior of merphalan derivatives in various systems.

Preliminary experiments showed that the probe (**V**) can be efficiently included into the liposomes prepared from egg yolk phosphatidylcholine and distributes in them rather uniformly at near 1% concentration. This is an important property for successful CFM experimentation with cells, which will be the subject of our subsequent communications.

EXPERIMENTAL

DCC, 4-dimethylaminopyridine (DMAP), triethylamine, and TFA were purchased from FLUKA (United States); *rac*-1-(*Z*-9-octadecenyl)glycerol (selachyl alcohol) from Supelco (United States); other reagents and solvents from Reakhim (Russia). Anhydrous chloroform was obtained by distillation over phosphorus pentoxide. Other solvents of domestic production were used after usual purification procedures. Column chromatography was performed on Kieselgel 60 (Merck, Germany) and alumina (Reakhim, Russia). Precoated plates Kieselgel 60 F_{254} and Kieselgel 60 (Merk, Germany) and reversed-phase Nano-Sil C_{18}-100 plates (Macherey-Nagel, Germany) were used for TLC. The spots were detected by (A) spraying with phosphomolybdic acid, (B) under UV irradiation, and (C) by treatment with Cl_2–benzidine. 3-(3-Perylenoyl)propionic acid [12] and *N*-Boc-sarcolysine [2] were obtained as described previously. Selachyl alcohol (a preparation stored for twenty years) was purified by column chromatography on alumina using 9 : 9 : 1 CH_2Cl_2–ethylacetate–methanol system for elution.

Mass spectra were taken on a MALDI time-of-flight Vision 2000 mass spectrometer (Thermobioanalysis, UK) using 2,5-dihydroxybenzoic acid as a matrix and N_2 laser (3 ns pulse, maximal pulse energy of 250 μJ) or an ESI time-of-flight Finnigan MAT 900S instrument (injection in chloroform–methanol 1 : 1). UV spectra were registered on a LKB Ultrospec II spectrophotometer (Sweden). Fluorescence spectra were taken on a Hitachi F-4000 spectrofluorimeter in 10 × 10 mm

quartz cuvettes at 20°C. ^{1}H NMR spectra (δ, ppm relative to TMS) were measured on a Bruker WM 500 spectrometer (Germany) in $CDCl_3$. Solvents were removed on a rotary evaporator in a vacuum at bath temperature below 40°C.

***rac*-1-(*Z*-9-Octadecenyl)-3-[3-(3-perylenoyl)propionyl]glycerol (III) and its 1,2-isomer.** A 50% solution of DCC in CCl_4 (100 μl) was added upon stirring to a solution of selachyl alcohol (**II**) (40 mg, 0.12 mmol), 3-(3-perylenoyl)propionic acid (30 mg, 0.085 mmol), and DMAP (40 mg, 0.33 mmol) in anhydrous chloroform (2 ml). The reaction mixture was kept for a day and treated with an additional DCC solution (50 μl; 75 mg, 0.36 mmol in total). Then the reaction mixture was diluted with CH_2Cl_2 (20 ml) and stirred with 1 N H_2SO_4 (1 ml) for 30 min at cooling with ice. The organic extract was twice chromatographed on a silica gel column (~1 × 13 cm) using gradient elution with ethyl acetate (5 ⟶ 15%) in pentane–CH_2Cl_2 (1 : 1) at TLC monitoring (45 : 45 : 10 pentane–CH_2Cl_2–ethylacetate, detection A, B, and C), to get fluorescent (**III**); yield 26 mg (45%); R_f 0.18; and, probably, 1,2-isomer of (**III**); yield 17 mg (30%); R_f 0.15 with the same fluorescence.

(**III**): amorphous orange substance; ESI-MS, *m*/*z*: 699.5 [*M* + Na]$^+$; UV [ethanol): λ_{max} 446.5 nm (ε 11500); ^{1}H NMR (δ, ppm): 0.89 (3 H, t, *J* 6.8 Hz, 3H, CH_3), 1.3–1.4 (24 H, m, H2–H7, H12–H17), 2.01 (4 H, m, H8, H11), 2.90 (2 H, t, *J* 6.4 Hz, 2H, CH_2COO), 3.44, 3.52 (6 H, 2 m, CH_2COAr, CH_2OCH_2), 4.05 (1H, s, C<u>H</u>OH), 5.35 (2 H, m, CH=CH); 7.54 (2 H, q), 7.61 (1 H, m), 7.74 (1 H, d), 7.79 (1 H, d), 7.99 (1 H, d), 8.22 (1 H, d), 8.27 (3 H, asymmetric t), 8.59 (1 H, d) (perylenyl); fluorescence: λ_{ex} 451 nm in ethanol and 459 nm in chloroform, λ_{em} 515 nm, λ_{em} 527 nm in ethanol and 511 nm in chloroform at λ_{ex} 450 nm, relative quantum yield 1.00.

1,2-Isomer: ESI-MS, *m*/*z*: 700.5 [*M* + Na]$^+$; fluorescence spectrum is the same as that of (**III**).

***rac*-1-(*Z*-9-Octadecenoyl)-2-(*N*-Boc-merphalanyl)-3-[3-(3-perylenoyl)propionyl]glycerol (IV).** A 50% solution of DCC in CCl_4 (0.29 mmol, 60 μl) was added to a stirred solution of (**III**) (10 mg, 15 μmol), *N*-Boc-merphalan (10 mg, 25 μmol), and DMAP (20 mg, 0.16 mmol) in anhydrous chloroform (0.7 ml). After a day, the reaction mixture was treated with DCC as described above and chromatographed on a silica gel column (0.5 × 7.5 cm) eluted with a 10 : 10 : 1 pentane–CH_2Cl_2–ethyl acetate system at TLC monitoring of the eluate (see above). The product (**IV**) was additionally filtered in CH_2Cl_2 through an alumina column (0.5 × 4 cm) for removal of dicyclohexylurea traces; yield 11 mg (70%); a red amorphous solid; R_f 0.45 (45 : 45 : 10 pentane–CH_2Cl_2–ethyl acetate, detection A, B, and C); ESI-MS, *m*/*z*: 1087.5 [*M* + Na]$^+$; UV: λ_{max} 445 nm (ε 12600); ^{1}H NMR (δ, ppm): 0.89 (3H, t, *J* 6.7 Hz, CH_3), 1.20–1.38 (24H, m, H2–H7 and H12–H17), 2.01 (4H, m, C<u>H</u>$_2$CH=), 2.87 (2H, t, *J* 7.3 Hz, CH_2COO), 2.97, 3.04 (2H, 2 m, CH_2Ar), 3.42, 3.49 (6H, 2 m, CH_2COAr, C<u>H</u>$_2$OCH$_2$CH$_2$), 3.52, 3.62 (10H, 2 m, NCH_2CH_2Cl, OC<u>H</u>$_2$CH$_2$), 4.24, 4.43 (2 H, 2 m, C<u>H</u>$_2$OOC), 4.56 (1 H, s, NH), 4.98 (1 H, m, C<u>H</u>NH), 5.25 (1H, m, C<u>H</u>OH), 5.34 (2H, s, CH=CH), 6.58, 7.04 (4 H, 2 m, phenylene), 7.54 (2H, m), 7.59 (1H, q), 7.74 (1H, d), 7.79 (1H, d), 7.98 (1H, q), 8.21 (1H, q), 8.27 (3H, asymmmetric t), 8.59 (1H, t) (perylenyl); fluorescence: λ_{ex} 450 nm in ethanol and 452 nm in chloroform, at λ_{em} 515 nm; λ_{em} 526 nm in ethanol and 506 nm in chloroform at λ_{ex} 450 nm; relative quantum yield 0.59.

***rac*-1-(*Z*-9-Octadecenyl)-2-merphalanyl-3-[3-(3-perylenoyl)propionyl]glycerol (V).** A solution of the amide (**IV**) (10 mg, 9.4 μmol) in CH_2Cl_2 (0.5 ml) and TFA (2 ml) was kept for 1 h at 35°C in argon atmosphere, and the reaction mixture was twice coevaporated with toluene. The residue was purified on a LiChroprep RP-18 (1 g) column eluted with a 95 : 4.5 : 0.5 methanol–CH_2Cl_2–TFA mixture to yield 7.8 mg (86%) of the amorphous red product (**V**); R_f 0.55 on Nano-Sil C_{18}-100 (89 : 10 : 1 methanol–CH_2Cl_2–TFA) and 0.45 on Kieselgel 60 without indicator (94 : 5 : 1 $CHCl_3$–isopropanol–CH_3COOH), detection with A, B, and ninhydrin; orange amorphous substance; MALDI MS, *m*/*z*: 965 [2M + H]$^+$; fluorescence: λ_{ex} 450 nm in ethanol and 452 nm in chloroform at λ_{em} 515 nm; λ_{em} 527 nm in ethanol and 507 nm in chloroform at λ_{ex} 450 nm; relative quantum yield 0.58.

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