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Effective Synthesis of a Novel Betulinic Acid Conjugate with Mitochondria-Targeting Cation F16 †

Darya Nedopekina, Eldar Davletshin and Anna Spivak *

Institute of Petrochemistry and Catalysis of Russian Academy of Sciences, 141 Prospekt Oktyabrya, 450075 Ufa, Russia; rawbe2007@mail.ru (D.N.); eldarik1996@mail.ru (E.D.)

* Correspondence: spivak.ink@gmail.com; Tel.: +7-9174-217-106

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Abstract: Currently, mitochondria are considered as an attractive universal target in the development of new anticancer drugs. These organelles are essential in energy production, regulation of cell death pathways, generation of reactive oxygen species, as well as maintenance of calcium homeostasis. Various approaches are being developed to deliver biologically active compounds into the mitochondria of tumour cells, including conjugation of cytotoxic substances with mitochondria-targeted lipophilic cations. Among the currently known low molecular weight lipophilic cationic molecules, (E)-4(1H-indol-3-ylvinyl)-N-methylpyridinium iodide (**F16**) is of great interest. This mitochondria-toxic cationic compound with luminescent properties is selectively accumulated in mitochondria and can selectively trigger apoptosis and necrosis of tumour cells, making it an attractive targeted agent for theranostic use. Meanwhile, betulinic acid, an available natural pentacyclic triterpenoid, has been considered as a promising scaffold for development of new anticancer agents in recent years. The antitumour effect of this natural product arises from affecting the mitochondria of tumour cells through formation of reactive oxygen species. The present article details an efficient synthesis of a novel multifunctional hybrid agent in which a cytotoxic triterpenoid, betulinic acid, is carbon-carbon bonded to the cationic **F16** fragment at the C-2 position of ring A through a phenylethynyl spacer. The starting substrates in the synthesis were C-2 propynyl derivative of betulinic acid and N-aryl-substituted 4-(1H-indol-3-ylvinyl)-pyridine. The derivative of betulinic acid with a terminal acetylenic group was prepared by the reaction of C-alkylation with propargyl bromide of potassium enoxytriethylborate generated from betulonic acid. To obtain the N-aryl-substituted analogue of **F16**, CuI-catalyzed Ullmann-Goldberg reaction was applied. The synthesis of the target conjugate was successfully completed by the cross-coupling of the terpene and heterocyclic components according to Sonogashira in the presence of CuI/$Pd(PPh_3)_2$ catalyst.

Keywords: betulinic acid; cross-coupling reaction; mitochondria; mitochondria-targeting cations; **F16**

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1. Introduction

The available plant metabolite, betulinic acid, and its semisynthetic derivatives represent an important class of biologically active substances and are in high demand in medicinal chemistry and pharmacological research (Figure 1). The antitumour effect of natural betulinic acid has been established in vitro against human tumour cells of various types [1,2]. This molecule, unlike many known cytostatics, directly affects the mitochondria of tumour cells, triggering the process of apoptosis of cancer cells [3,4]. The antitumour activity of betulinic acid combines well with low systemic toxicity. However, the poor bioavailability of this triterpene, associated with poor solubility in an aqueous medium, prevents reaching the target in vivo and achieving the desired therapeutic effect [5,6]. In recent years, conjugation of natural triterpene acids with cationic lipophilic

molecules with low molecular weight has been efficiently applied to enhance the bioavailability and antitumour activity [7]. These positively charged small molecules easily penetrate into mitochondria due to the large value of the membrane potential of mitochondria compared to the potential of the cell membrane (ΔΨmito = 150–180 mV, ΔΨplasma = 30–60) [8,9]. The prospects of involving mitochondria-targeted cationic fragments as a "vector" for selective delivery of cytotoxic triterpenoids into the mitochondria of cancer cells have been demonstrated in the study of conjugates of triterpenic acids with triphenylphosphonium cation or with rhodamine B [10].

Recently, a novel mitochondria-toxic cationic compound (*E*)-4(1H-indol-3-ylvinyl)-*N*-methylpyridinium iodide (**F16**) was discovered (Figure 1).

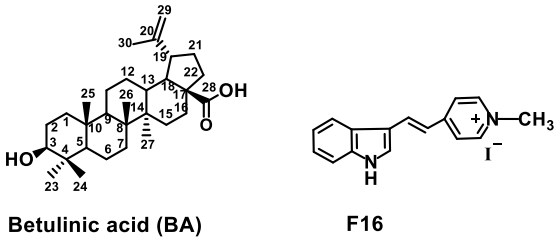


Figure 1. Structures of betulinic acid (**BA**) and compound **F16**.

This delocalized lipophilic cation is selectively accumulated in the mitochondrial matrix of various tumour cell lines [11,12]. Its high concentration in mitochondria results in cell death associated with the arrest of the cell cycle, interruption of the mitochondrial respiratory chain, a decrease in the intracellular level of ATP, and the induction of apoptosis. The fluorescent properties of **F16** offer good prospects for application of this compound as a fluorescent probe for imaging tumours. Furthermore, hybridization of cytotoxic agents with this delocalized cation can contribute to the development of new theranostic agents for cancer therapy. Thus far, however, unlike the triphenylphosphonium cation widely known today, only a few studies have been reported on the potential of **F16** as a means of delivering biologically active compounds to malignant transformed cells [13,14].

Earlier, we reported on the first synthesis of conjugates of triterpene acids with a fragment of the cationic molecule **F16** in the work [15]. In the tests involving different tumour cell lines, the new hybrid compounds exhibited significantly higher cytotoxicity (≈100–200 times) than the initial betulinic acid, along with acceptable selectivity in the relationship between tumour and healthy cells. It is noteworthy that the **F16** pharmacophore fragment in the resulting conjugates was linked to the 3-OH or 17-COOH groups of the triterpene nucleus through an ester function, which may be unstable to the action of enzymes under biochemical conditions. In this regard, here we detail development of an effective approach to the synthesis of a novel hybrid molecule "triterpenoid—F16", in which the nitrogen atom of the F16 indole ring is linked to the A ring of betulinic acid at the C-2 position through a phenylethynyl spacer.

2. Materials and Methods

2.1. Chemistry

The starting compounds betulinic acid and reagents: BEt_3 (95%), $KN(SiMe_3)_2$ (potassium bis(trimethylsilyl)amide, 1 M solution in THF), propargyl bromide, DME (dimethoxyethane), pyridine-4-carbaldehyde, Tri-n-butylphosphine, 1,4-diiodobenzene, piperidine-2-carboxylic acid, CuI, DMF (dimethylformamide), $PdCl_2(PPh_3)_2$, CuI, Et_3N, CH_3I were purchased from Acros Organics (Geel, Belgium) and used without any further purification. Synthesizes and spectral data of compounds **1, 2, 3a, 5** and **F16a** have been published as previously reported [16–19].

2.1.1. Synthesis of 1-Iodo-4-{(E)-4-[2-(1H-indol-3-yl)vinyl]-pyridine}phenyl (**3**).

The 1,4-diiodobenzene (660 mg, 2 mmol) was added to a suspension of (E)-4-[2-(1H-indol-3-yl)vinyl]-pyridine (220 mg, 1 mmol), K_2CO_3 (105 mg, 0.8 mmol), CuI (12 mg, 0.06 mmol), piperidine-2-carboxylic acid (15 mg, 0.12 mmol) in dry DMF (5 mL) and stirred at 110 °C for 24 h. Then the mixture was cooled to room temperature and evaporated under reduced pressure. The residue was chromatographed on silica gel, using hexane/EtOAc (from 15:1 to 1:1) and recrystallized wih EtOAc to give pure product **3** as a orange-yellow powder (211 mg, 0.5 mmol, 50%). IR (film) ν_{max} 1633 (CH=CH), 1593, 1491, 1456 (Ph) cm^{-1}; $[\alpha]_D^{20}$ 0° (c 0.17, $CHCl_3$); m.p. 186–188 °C (EtOH); ^{1}H-NMR (500 MHz, MeOD): δ 8.43 (2H, br s, H-17, H-21), 7.98 (1H, br s, H-10), 7.81 (2H, d, J = 6.5 Hz, H-3, H-5), 7.54–7.20 (9H, m, H-2, H-6, H-7, H-11, H-12, H-13, H-18, H-20 and H-15 or H-16), 7.03 (1H, d, J = 16.5 Hz, H-15 or H-16) ppm; ^{13}C-NMR (125 MHz,$CDCl_3$/MeOD): δ 149.4 (C-17, C-21), 147.0 (C-19), 139.2 (C-3, C-5), 138.9 (C-14), 136.8 (C-1), 128.6 (C-15 or C-16), 127.2 (C-9), 126.7 (C-7), 126.4 (C-2, C-6), 124.0, 123.0, 122.1, 121.0, 120.7 (C-10, C-11, C-12, C-15 or C-16, C-18 and C-20), 116.1 (C-8), 111.2 (C-13), 91.8 (C-4) ppm; Anal Calcd. for $C_{21}H_{15}IN_2$: C, 59.73; H, 3.58. Found: C, 60.16; H, 4.12; MS (HRMS): m/z calcd. for $C_{21}H_{15}IN_2$ $[M+H]^+$ 423.0353; found 423.0330.

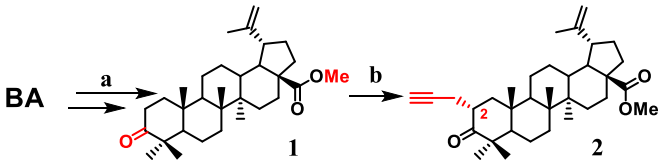
2.1.2. Synthesis of Methyl 2α-{[(E)-4-(1H-indol-3-yl-vinyl)-N-methyl-pyridinium iodide]phenylpropynyl}-3-oxolup-20(29)en-28-oate (**4**).

A mixture of triterpenoid **2** (110 mg, 0.2 mmol), iodophenyl derivative **3** (84.5 mg, 0.2 mmol), $PdCl_2(PPh_3)_2$ (7.0 mg, 0.01 mmol) and CuI (3.8 mg, 0.02 mmol) were dissolved in anhydrous Et_3N/DMF (4 mL, 1:1). The resulting mixture was stirred at room temperature for 1 h under an argon atmosphere, until starting material was observed by TLC. Then reaction was quenched by addition of water and extracted with EtOAc (3 × 10 mL). The combined organic extracts were dried with $MgSO_4$ and concentrated under reduced pressure. To a solution of crud light brown product (86 mg, 0.1 mmol) in dry DMF (2 mL) CH_3I (0.06 mL, 1 mmol) was added and stirred at room temperature in argon atmosphere for 12 h. Then the solvent evaporated under reduced pressure. The residue was chromatographed on silica gel, using CH_2Cl_2/MeOH (from 30:1 to 10:1), to obtain pure product **3** as a orange powder (67 mg, 0.07 mmol, 71%). IR (film) ν_{max} 1716 (C=O), 1636 (CH=CH), 1609, 1509, 1458 (Ph) cm^{-1}; $[\alpha]_D^{19}$ –13.3° (c 0.06, $CHCl_3$); m.p. 196–198 °C (EtOH); ^{1}H-NMR (500 MHz, MeOD): δ 8.49 (2H, d, J = 6.5 Hz, H-20′, H-24′), 8.09–8.07 (2H, m, H-13′, H-18′ or H-19′), 8.00–7.96 (3H, m, H-21′, H-23′, H-10′), 7.52–7.44 (5H, m, H-5′, H-6′, H-8′, H-9′, H-16′), 7.33–7.31 (2H, m, H-14′, H-15′), 7.20 (1H, d, J = 16.0 Hz, H-18′ or H-19′), 4.80 (3H, s, N^+CH_3), 4.72, 4.58 (2H, both br s, H-29), 3.68 (3H, s, OCH_3), 3.01–2.99 (2H, m, H-2, H-19), 2.80 (1H, dd, J = 15.0, 5.0 Hz, H^a-1′), 2.48–2.40 (2H, m, H^a-1, H^b-1′), 2.29–1.13 (21H, m, CH, CH_2 in pentacyclic skeleton), 1.68 (3H, s, H-30), 1.19, 1.10, 1.09, 1.01, 1.00 (all s, 3H each, H-23–H-27) ppm; ^{13}C-NMR (125 MHz,$CDCl_3$): δ 215.8 (C-3), 176.6 (C-28), 154.3 (C-22′), 150.5 (C-20), 143.9 (C-20′, C-24′), 137.1 (C-7′, C-17′), 135.8 (C-18′ or C-19′), 133.4 (C-10′), 133.1 (C-5′, C-9′), 126.4 (C-12′), 124.2 (C-14′), 124.0 (C-6′, C-8′), 122.8 (C-15′), 122.7 (C-21′, C-23′), 121.1 (C-13′), 118.1 (C-18′ or C-19′), 115.4 (C-11′), 111.5 (C-16′), 109.7 (C-4′, C-29), 90.5 (C-2′), 80.6 (C-3′), 57.4 (C-5), 56.5 (C-17), 51.3 (OCH_3), 50.1 (C-9), 49.4 (C-18), 48.4 (C-4), 47.8 (N^+CH_3), 47.00 (C-1, C-19), 42.5 (C-14), 41.7 (C-2), 40.8 (C-8), 38.2 (C-13), 37.5 (C-10), 36.9 (C-22), 34.1 (C-7), 32.1 (C-16), 30.5 (C-21), 29.6 (C-15), 25.4 (C-12), 25.1 (C-23), 21.7 (C-25), 21.2 (C-11), 20.6 (C-1′), 19.4 (C-30), 19.3 (C-6), 16.2 (C-24), 16.1 (C-26), 14.7 (C-27) ppm; Anal. Calcd for $C_{56}H_{67}IN_2O_3$: C, 71.32; H, 7.16. Found: C, 71.26; H, 7.19; MS (HRMS): calcd for $C_{56}H_{67}N_2O_3$ $[M–I]^+$ 815.5146; found: 815.5176.

3. Results and Discussion

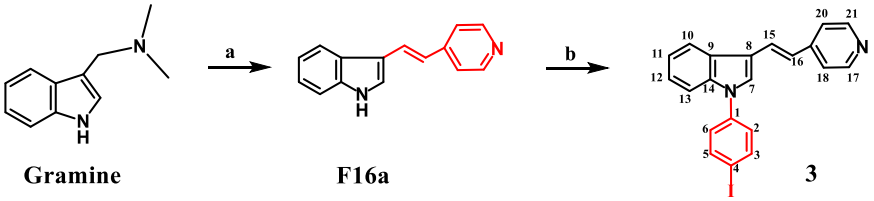
In the synthesis of the target conjugate **4**, the C-2 propargyl derivative of betulinic acid **2**, prepared from betulinic acid in several stages according to the method previously developed by us [16], was used as the starting compound. The key stage of the scheme is

alpha-alkylation with propargyl bromide of potassium enoxytriethylborate generated from methylbetulonate **1** under the action of $KN(SiMe_3)_2$-Et_3B (Scheme 1).



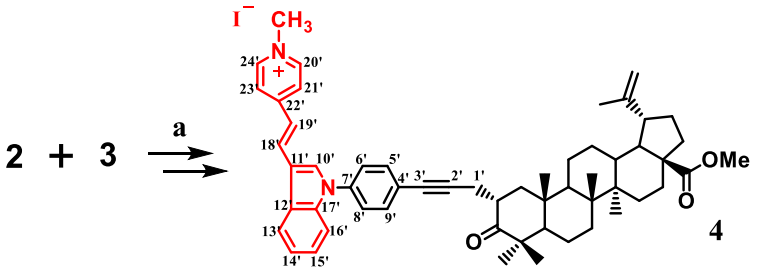
Scheme 1. Synthesis of C-2 propynyl derivative **2**. *Reagents and conditions:* **a.** *1.* CrO_3, H_2SO_4, $(CH_3)_2CO$, 2 h; *2.* CH_2N_2, Et_2O, 0 °C; **b.** $KN(SiMe_3)_2$, Et_3B, C_3H_3Br, DME, Ar, 2 h.

The iodophenyl derivative of (E)-4-(1H-indol-3-ylvinyl)-pyridine **3** was synthesized as the second component to obtain conjugate **4**. Heterocyclic compound **F16a** was obtained by the reaction of gramine with pyridine-4-carbaldehyde involving tri-n-butylphosphine as described in [20]. The CuI-catalyzed coupling reaction of **F16a** according to Ullmann-Goldberg with a two-fold excess of 1,4-diiodobenzene gave compound **3** in 50% yield (^1H and ^{13}C NMR spectra) (Scheme 2).



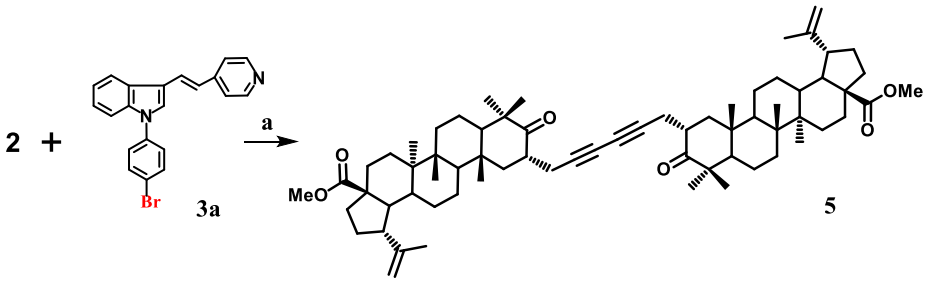
Scheme 2. Synthesis of compound **3**. *Reagents and conditions:* **a.** pyridine-4-carbaldehyde, tri-n-butylphosphine, CH_3CN, 81 °C, Ar, 24 h; **b.** 1,4-diiodobenzene, piperidine-2-carboxylic acid, CuI, K_2CO_3, DMF, 110 °C, Ar, 24 h.

Conjugate **4** was prepared by the Sonogashira cross-coupling reaction of triterpenoid **2** with heterocyclic compound **3** in the presence of $CuI/Pd(PPh_3)_2$ catalyst in Et_3N/DMF solvent mixture. The resulting adduct was transformed into pyridinium derivative without preliminary purification by quaternization of the pyridinium ring under the action of CH_3I in DMF (Scheme 3). The reaction proceeded at room temperature for 12 h producing the only product, the target hybrid compound **4**, in 71% yield (^1H and ^{13}C NMR spectra).



Scheme 3. Synthesis of C-2 conjugate of betulinic acid—**F16 4.** *Reagents and conditions:* **a.** *1.* $PdCl_2(PPh_3)_2$, CuI, Et_3N/DMF (1:1), Ar, 2 h; *2.* CH_3I, DMF, 12 h.

It should be pointed out that our experiments to involve the **F16a** derivative containing a bromophenyl substituent at the nitrogen atom of the indole ring (compound **3a**) in the Sonogashira reaction failed. In this case, the reaction proceeded only through acetylenic homodimerization of triterpenoid **2** giving a single product **5** (Scheme 4).



Scheme 4. Synthesis of compound **5.** *Reagents and conditions:* **a.** $PdCl_2(PPh_3)_2$, CuI, Et_3N/DMF (1:1), Ar, 2 h.

The structure of the resulting conjugate **4** was specified applying 1D (1H, 13C, APT) and 2D homo-(COZY, NOESY) and heteronuclear (HSQC, HMBC) NMR experiments. Nuclear-chemical shifts for the terpene nucleus and for *(E)*-4(1H-indol-3-ylvinyl)-*N*-methylpyridinium iodide were determined by comparison with previously published data [16,18]. In the ^{1}H NMR spectra, the presence of the fragment (*E*)-4(1H-indol-3-ylvinyl)-*N*-methylpyridinium iodide was confirmed by the characteristic signal for the pyridinium ring as a doublet at 8.49 ppm, J = 6.5 Hz, as well as a doublet of the vinyl group at 7.21 ppm, J = 16.0 Hz, a singlet of the methyl group at 4.80 ppm (N^+CH_3) and four multiplets characteristic of the indole and phenyl fragments at 8.09–8 07, 8.00–7.96, 7.52–7.44 and 7.33–7.30 ppm. One of the protons of the methylene group H^a-1′ resonated as a doublet of doublets, J = 15.0, 5.0 Hz, the second proton H^b-1′ and proton H^a-1 appeared as a multiplet in the range of 2.48–2.40 ppm. Signals of carbon atoms characteristic of (*E*)-4(1H-indol-3-ylvinyl)-*N*-methylpyridinium iodide and phenyl ring in the range of 154.3–109.7 and 47.8 ppm were registered in the ^{13}C NMR spectra. The signals of the C-2′ and C-3′ carbon atoms resonated at 90.5 and 80.6 ppm, respectively.

4. Conclusions

Thus, we have developed an effective approach to produce a conjugate of the cytotoxic triterpenoid of betulinic acid with **F16**, carrying a fragment of a cationic compound as a vector for the delivery of a hybrid molecule into the mitochondria of tumour cells. We believe that this modification of betulinic acid will enhance its bioavailability and antitumour activity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

Author Contributions: Validation and writing—review and editing, A.S.; performing the chemistry experiments, D.N. and E.D.. The manuscript was prepared through the contributions of D.N. and E.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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