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Regioselective synthesis of folate receptor-targeted agents derived from epothilone analogs and folic acid

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ABSTRACT

Efficient regioselective syntheses of conjugates of folic acid and cytotoxic agents derived from natural epothilones are described. These folate receptor (FR) targeting compounds are water soluble and incorporate a hydrophilic peptide-based spacer unit and a reducible self-immolative disulfide-based linker system between the FR-targeting ligand and the parent drug.

The major aims of targeted chemotherapies are twofold: to improve the efficacy of currently available methods and to reduce the inherent collateral toxicity to normal tissues. Receptor-specific targeting is an innovative approach that can potentially herald a new era in selective delivery of cytotoxic drugs to pathologic cells.

Folic acid (FA) binds with a high affinity cell-surface glycoprotein called the folate receptor. After binding, FA is transported into the cell via FR-mediated endocytosis.[1] The FR is expressed at relatively high levels in human epithelial cancers, but has limited expression in normal tissues. Additionally, conjugation of biologically active compounds to FA does not significantly affect FR binding and FR-mediated endocytosis in a vast number of cases. Consequently, FA can be exploited as a molecular 'Trojan horse' for the targeted delivery of covalently-attached, biologically active molecules.[2] In pursuing this strategy, scientists at Endocyte have synthesized a series of FA conjugates of single chemotherapeutic drugs, including desacetyl-vinblastine hydrazide, mitomycin, and tubulysin; as well as a dual drug combination of mitomycin and desacetyl-vinblastine hydrazide.[3]

Epothilones (Epo)[4] are 16-membered macrolides. Their activity, like that of paclitaxel, is based on the inhibition of cell division by binding to microtubules resulting in their stabilization. Microtubules are responsible for various cellular activities, such as mitosis, motility and trafficking of vesicles, organelles and proteins. Agents which disrupt the dynamics of microtubules, can induce apoptosis.[5] On account of their higher affinity, the epothilones displace paclitaxel from microtubules and show activity against cancer cells resistant to paclitaxel.[6] Several epothilones and epothilone analogs have entered human clinical trials, with ixabepilone being the first drug in this class to receive FDA approval.[7]

In this Letter, we report the design and regioselective synthesis of FA–Epo conjugates **1a–c** (Scheme 1). As indicated in the retrosynthetic analysis, **1** can be assembled by tethering a FA-spacer unit **2** to epothilone derivatives (**3a–c**) via a self-immolative linker system containing a reducible disulfide bond, which is important for drug delivery applications. A recent study involving real-time imaging using a fluorescence resonance energy transfer technique has demonstrated that reduction-mediated release of the drug cargo from a disulfide linked FA-conjugate efficiently occurs within the endosomes of cancer cells.[8]

The peptide-based spacer **2** was designed to be bifunctional containing both acidic (Asp) and basic (Arg) amino acids to provide solubility of the final drug conjugate under physiological conditions. This unit was assembled using standard fluorenylmethyloxycarbonyl-based solid phase peptide synthesis (Fmoc SPPS).[3]

Activated carbonate **5**[3] (Scheme 2) served as universal heterobifunctional crosslinker for the preparation of all conjugates described below. The synthesis of our first folate conjugate involved 21-amino-epothilone B **4** as the parent drug. As shown in Scheme 1, the amine **4** was treated with the activated carbonate **5** and diisopropylethylamine (DIPEA) in dichloromethane to yield 2-pyridyldisulfanylethyl carbamate **3a**. After chromatographic purification on silica gel, **3a** was isolated in 80% yield. Treatment of a

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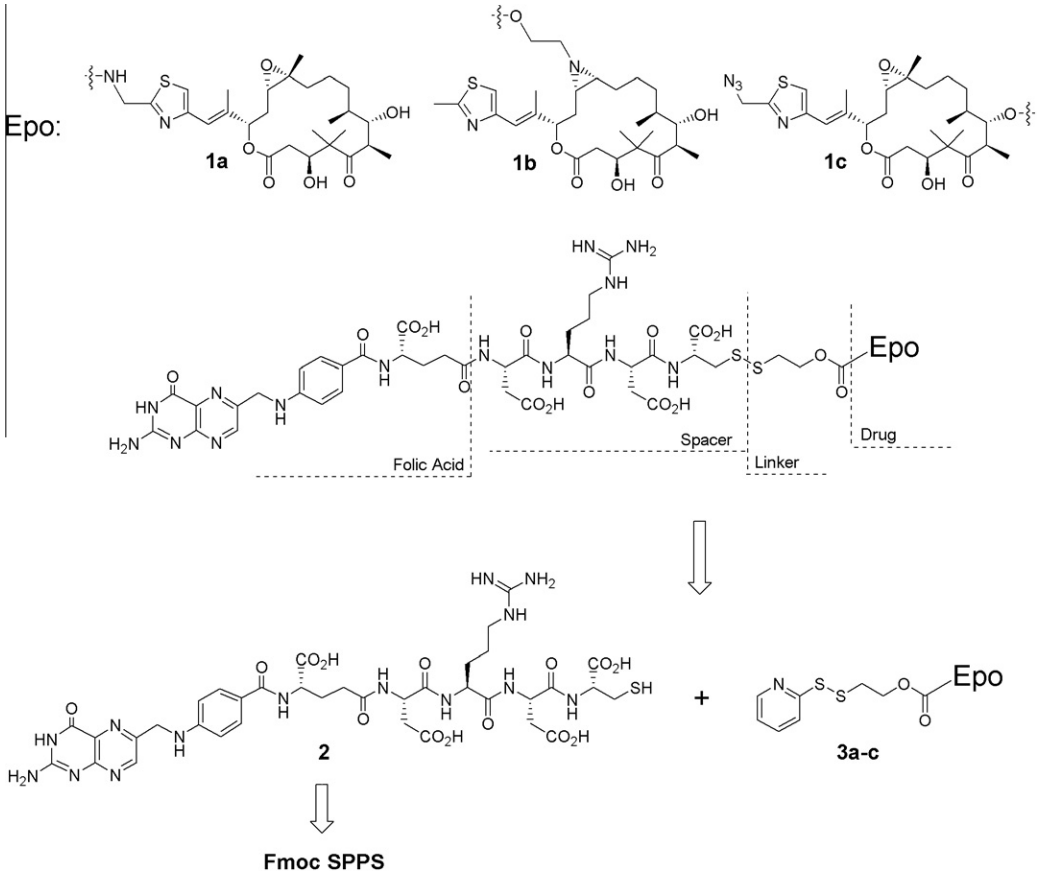
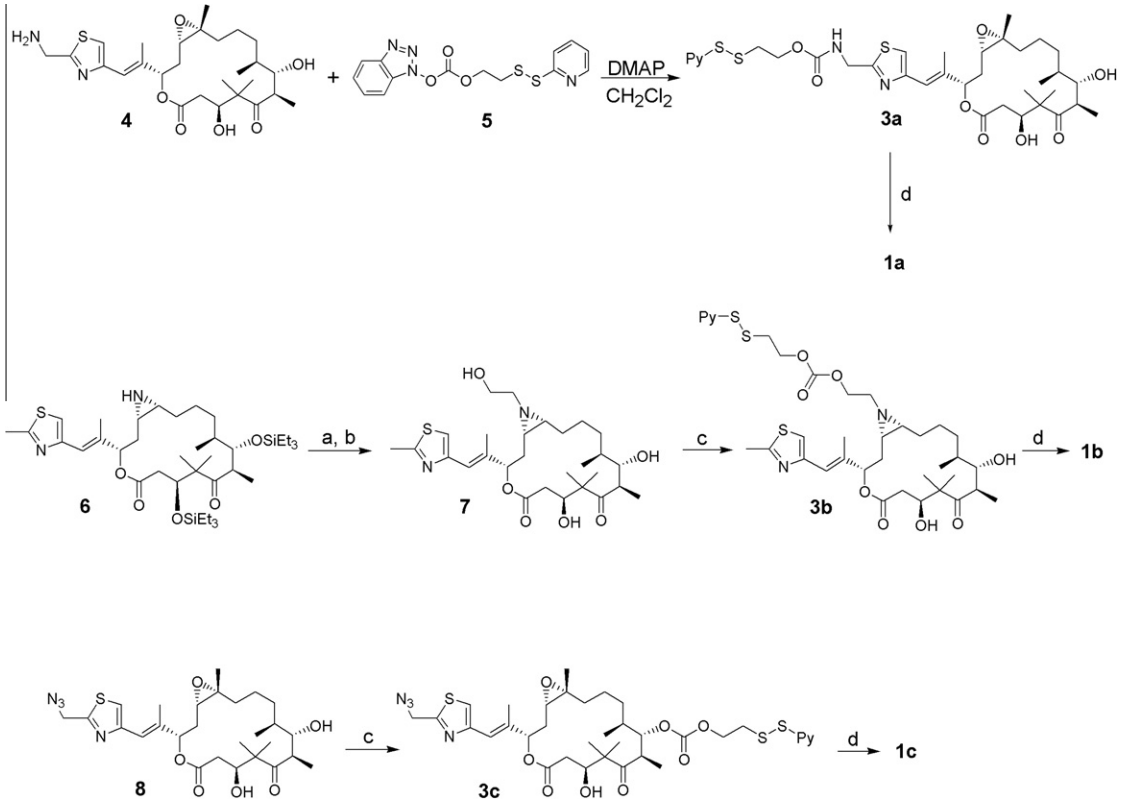
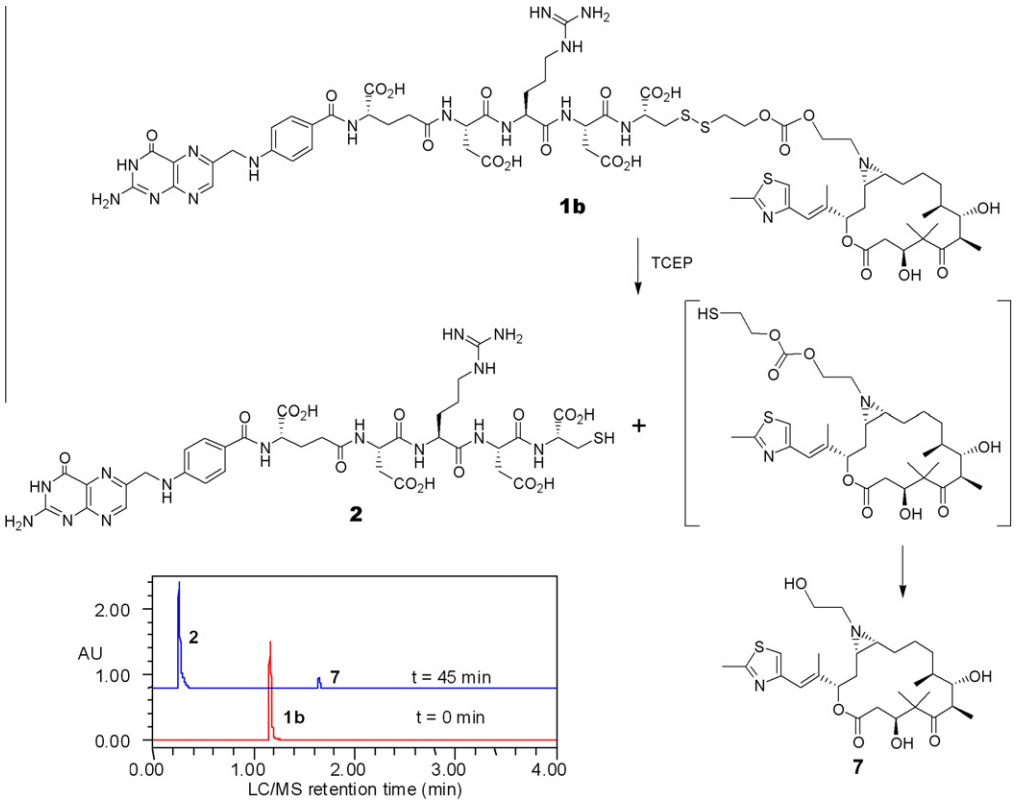


Scheme 1.



Scheme 2. Reagents and conditions: (a) 2-bromoethanol, K_2CO_3, acetonitrile, 80 °C; (b) TFA/CH_2Cl_2; (c) **5**, DMAP, CH_2Cl_2; (d) **2**, H_2O, THF.



Scheme 3. Conjugate **1b** in 50 mM NH_4OAc buffer (pH 5.5) treated with TCEP.

suspension of FA-spacer **2** in H_2O under argon with 0.1 N $NaHCO_3$ resulted in a clear yellow solution at pH >6.5. To this mixture was added at once under extensive stirring a solution of **3a** in THF. According to the HPLC profile, the reaction was completed in less then 30 min.[9] HPLC purification gave pure conjugate **1a**.

SAR[10] studies performed on semisynthetic derivatives of epothilones showed that some N-substituted derivatives of 12α,13α-aziridinyl epothilone are more potent than the natural compounds, so we decided to modify the aziridine moiety by simple N-alkylation. Thus, treatment of suitably protected 12α,13α-aziridinyl epothilone A **6**[10] with 2-bromoethanol and K_2CO_3 in acetonitrile, followed by deprotection of TES in TFA/CH_2Cl_2, resulted in a novel member of the epothilone family, namely *N*-hydroxyethyl-12α,13α-aziridinyl epothilone A **7**.[11] Its cytotoxicity profile shows that it is among the most potent epothilones. The primary hydroxyl group of **7** was reacted with activated carbonate **5** to provide **3b**.[12,13] This thiophilic pyridyldisulfanyl-epothilone derivative was treated with the thiol-containing folate spacer unit **2** as described above[8] to give the desired product **1b** (Epofolate, BMS-753493). LC/MS and ^1H NMR data[14] were in agreement with the expected structure.

The next Epo–FA conjugate involved the modification of the sterically hindered C7-hydroxyl group in the epothilone macrolide skeleton. As shown by electron crystallography studies,[15] the C7-hydroxyl group in the molecular architecture of epothilones is proposed to be part of the hydrogen-bonding network responsible for the specific binding to β-tubulin. Successful implementation of our conjugation methodology to the C7–OH would open a wide range of epothilones to folate conjugation, rather than being limited to only those epothilones with more accessible nucleophilic moieties.[16] 21-Azido-epothilone B **8** was reacted with excess of cross-linker **5** in the presence of DMAP to give carbonate **3c** as a single product. The regioselectivity of this modification was confirmed by ^1H NMR: the signal for the proton attached to C7-atom shifted downfield from 3.76 ppm in **8** to 5.18 ppm in **3c**. Treatment of **3c** with folate spacer unit **2** resulted as expected in clean conversion to conjugate **1c**.

A model study that mimics the release of free drug within the reductive environment in the endosome was performed on conjugate **1b**. We performed our chemical release study at pH 5.5 (50 mM NH_4OAc buffer) to match the acidic environment in endosome.[17] A 1 mM solution of **1b** was treated with 5 mM of tris(2-carboxyethyl)phosphine (TCEP) at 37 °C. The HPLC profile (Scheme 3, UV detection at 280 nm) showed complete cleavage of the disulfide bond with concomitant release of the parent drug **7** and the FA-spacer **2** within 45 min. These results were confirmed by LC/MS.

In summary, we described the expedient and regioselective synthesis of folate conjugates of a structurally diverse group of epothilones and demonstrated the release of the parent drug from one of the intact conjugates in a simple model study. Furthermore, we presented the new and powerful epothilone derivative **7**. We also were able to react successfully the sterically hindered C7–OH in the epothilone backbone with our heterobifunctional linker **5**, so that the resulting pyridyldisulfanylethyl carbonate can be used as precursor for folate conjugation. Compound **1b** (Epofolate, BMS-753493) is in phase I clinical trials for solid tumors. Folate–epothilone conjugates are being tested against a variety of FR positive cell lines as well as in animal models. The results of the complex biological investigations will be reported in appropriate scientific journals.

Acknowledgment

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9. *General procedure for conjugation:* To H_2O (bubbled with argon for 10 min before use) was added folate linker in a centrifuge tube. To this suspension, while bubbling with argon, was added dropwise saturated $NaHCO_3$ solution (bubbled with argon for 10 min before use) until the pH of the resulting solution reached 6.9. One equivalent of the epothilone carbonate in THF was added quickly and the resulting homogenous solution was stirred under argon for 30 min. The reaction progress was checked by analytical HPLC. The mixture was diluted with of phosphate buffer and the THF was removed under vacuum. The cloudy solution was centrifuged and filtered. The yellow filtrate was purified by preparative HPLC.
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11. K_2CO_3 (1.4 g, 10.2 mmol) and 2-bromoethanol (0.52 mL, 7.3 mmol) were added to solution of **6** (1.05 g, 1.46 mmol) in acetonitrile (20 mL) and heated to 80 °C. After 4 h, additional 2-bromoethanol (0.52 mL, 7.3 mmol) and K_2CO_3 (1.4 g, 10.2 mmol) were added. After 5 h, additional 2-bromoethanol (0.21 mL, 2.92 mmol) was added. After 3 h, the reaction mixture was cooled to room temperature, filtered through Celite, washed with acetonitrile (5 × 5 mL), dichloromethane (2 × 5 mL), concentrated and taken to next step without further purification.
12. *General procedure for preparing of drug carbonates:* To a solution of epothilone derivative in anhydrous dichloromethane at 0 °C was added DMAP (1.2 equiv) and **5** (1.0 equiv). The reaction mixture was stirred at 0 °C under argon and monitored by TLC. Additional DMAP (1.2 equiv) and **5** (1.0 equiv) were added as necessary until all of epothilone derivative was consumed. The reaction was quenched with MeOH at 0 °C, the solvent was removed under vacuum, and the residue was purified by chromatography (silica gel, 2.5–5% MeOH in DCM) to afford the epothilone carbonate derivative.
13. ^{1}H NMR data for compound **3b** (acetone-d_6, 300 MHz): δ 8.46–8.44 (m, 1H, py-H), 7.81–7.79 (m, 2H), 7.24–7.19 (m, 2H, py-H+Epo-H), 6.59 (s, br, 1H), 5.44–5.41 (m, 1H), 4.89–4.86 (m, 1H), 4.40 (t, 2H, J = 6.4 Hz), 4.36–4.23 (m, 2H), 4.19–4.12 (m, 1H), 3.77–3.71 (m, 1H), 3.52–3.49 (m, 1H), 3.25 (dd, 1H, J = 8.5, 6.8 Hz), 3.16 (t, 2H, J = 6.1 Hz), 3.13–3.08 (m, 1H), 2.67 (s, 3H, CH_3), 2.64–2.51 (m, 4H), 2.17 (s, 3H, CH_3), 2.04–1.93 (m, 1H), 1.88–1.78 (m, 2H), 1.66–1.25 (m, 8H+CH_3), 1.13 (d, 3H, J = 6.7, CH_3 Hz), 1.04 (s, 3H, CH_3), 0.9 (d, 3H, J = 6.7 Hz, CH_3).
14. *Analytical data for* ***1b*** *(Epofolate):* HRMS calcd: 785.29454 $(M+2H)^{2+}$, 523.86563 $(M+3H)^{3+}$, 393.15118 $(M+4H)^{4+}$; found: $(M+2H)^{2+}$ at 785.29100 (4.5 ppm), $(M+3H)^{3+}$ at 523.86431 (2.5 ppm), $(M+4H)^{4+}$ at 393.14996 (3.1 ppm).
^{1}*H NMR data (D2O, 300 MHz):* δ 8.55 (s, 1H, FA H-7), 7.47 (d, 2H, J = 8.8 Hz, FA H-12 and 16), 6.93 (s, 1H), 6.57 (d, 2H, J = 8.8 Hz, FA H-13 and 15), 6.31 (s, 1H), 5.12–5.09 (m, 1H), 4.54–4.48 (m, 1H), 4.43–4.39 (m, 2H), 4.24–4.12 (m, 4H), 3.98–3.93 (m, 1H), 3.52–3.50 (m, 1H), 3.22–3.08 (m, 2H), 3.00–2.92 (m, 3H), 2.80–2.40 (m, 5H + CH_3), 2.33–2.21(m, 3H), 2.04–1.86 (m, 3H), 1.76–1.54 (m, 1H+ CH_3), 1.46–1.33 (m, 2H), 1.19 (s, 3H, CH_3), 1.00 (d, 3H, J = 6.4 Hz, CH_3), 0.85(s, 3H, CH_3), 0.82 (d, 3H, J = 5.9 Hz, CH_3).
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