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Macromolecular prodrugs XI. Synthesis and characterization of polymer–estradiol conjugate

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Abstract

Estradiol-3-benzoate (EB), an ester derivative of the main oestrogen hormone estradiol, was chemically modified and bound to poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))–poly(α,β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer (PAHA). EB was first converted to estradiol-3-benzoate-17-(benzotriazole-1-carboxylate), which readily reacted with amino groups in PAHA affording the polymer–drug conjugate PAHA–EB. In PAHA–EB estradiol moiety was covalently bound to the polymeric carrier by carbamate linkage, through non-toxic ethylenediamine spacer. The synthesized compound is a potential hydrosoluble estradiol prodrug.

Keywords: Estradiol; Estradiol benzoate; Poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))–poly(α,β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer; Polymer–drug conjugate; Macromolecular prodrug

1. Introduction

A promising approach to improve drug delivery is to link an active agent as a side substituent to a polymeric backbone by means of a cleavable bond (Duncan, 2002; Duncan, 2003; Giammona et al., 1995; Giammona et al., 1998; Vasey et al., 1999). Such macromolecular prodrugs (polymer–drug conjugates) may offer many advantages compared to other drug delivery systems such as increased drug solubility, prolonged drug release and increased stability. It is also possible to

Abbreviations: Bt, 1-benzotriazolyl; Btc, 1-benzotriazolylcarbonyl; DMF, *N*,*N*′-dimethylformamide; DSC, differential scanning calorimetry; E, stradiol; EB, estradiol benzoate; NMR, nuclear magnetic resonance; PAHA, poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))–poly(α,β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer; PAHA–EB, PAHA-estradiol-3-benzoate conjugate; TGA, thermogravimetric analysis; TEA, triethylamine

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accumulate drug at the site of the pathological process and to minimize its toxicity on normal tissue.

In author's previous papers, the use of poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide)) (PHEA), poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))-poly(α, β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer (PAHA) and poly(α,β-(*N*-3-hydroxypropyl-DL-aspartamide)) (PHPA) as drug carriers for carboxylic acid, amino acid and amino drugs was described (see for example, Zorc and Butula, 1994; Kalčić et al., 1996; Lovrek et al., 2000; van der Merwe et al., 2002). In this paper we report synthesis and characterization of estradiol-3-benzoate-17-(benzotriazole-1-carboxylate) (**2**) and PAHA–estradiol conjugate in which estradiol-3-benzoate is covalently linked to PAHA, a polyaspartamide polymer bearing both hydroxyl and amino groups (Zorc et al., 1995). PAHA–estradiol conjugate is a potential hydrosoluble polymeric prodrug. Benzotriazole derivative (**2**) is also a potential estrogen prodrug. Analogous imidazole 1-carboxylic acid esters of estradiol 3-methyl ether, 5α-estran-17β-ol-3-one) and several other steroidal alcohols were found to have endocrinological activities equal to or slightly less than the parent steroids (Fahrenholtz et al., 1974).

2. Materials and methods

2.1. Spectroscopic measurements

2.1.1. NMR

All one (^{1}H and APT), and gradient-selected two-dimensional (gCOSY, gHSQC and gHMBC) NMR spectra were recorded at ambient temperature on a Bruker Avance DRX500 spectrometer operating at 500.13 MHz for ^{1}H and equipped with 5 mm diameter inverse detection probe and *z*-gradient. Sample concentration in DMSO d_6 was 20 mg ml^{-1}. TMS was used as the internal standard.

Typical spectral conditions for one-dimensional ^{1}H and ^{13}C (APT) spectra were as follows: the spectra were recorded using 64 K data points and spectral widths of 7700 Hz and 32,000 Hz for ^{1}H and ^{13}C experiments, respectively; digital resolution was 0.12 Hz and 0.48 Hz per point, respectively. The number of scans was 16 for ^{1}H and 1000 for APT spectra.

2D gradient selected COSY spectra and TOCSY spectra were acquired with the sweep width of 6600 Hz in both dimensions using 2 K data points and 512 increments. Digital resolution was 6.50 Hz per point in both dimensions. TOCSY spectra were obtained using the spin-lock time of 60 ms.

The gradient selected inverse ^{1}H–^{13}C correlation experiments, gHSQC and gHMBC were recorded at 125.77 MHz using the acquisition matrix of 1 K × 256 with 32 scans and processed with 2 K × 1 K transformed matrix. The sweep width was 6600 Hz in f2 dimension and 32000 Hz in f1 dimension for both experiments. Digital resolution was 3.25 Hz per point and 30.70 Hz per point in f2 and f1, respectively. HMBC spectra were recorded using transfer delay for the evolution of long-range C–H couplings of 60 ms.

2.1.2. IR and UV

IR spectra were recorded on a FT-IR Paragon 500 spectrometer (Perkin-Elmer, UK) and UV spectra on a Hewlett Packard 8452A Diode Array spectrophotometer (Hewlett Packard, Germany).

2.2. Thermogravimetric measurements

2.2.1. DSC

DSC thermograms were obtained using Pyris 1 DSC instrument, Perkin-Elmer (USA). The instrument was calibrated with indium and zinc prior analysing the samples under nitrogen purge of 35 ml min^{-1}. Samples were scanned in closed aluminium pans at a heating rate of 2 °C min^{-1} over the temperature range from 30 to 250 °C. The data were analyzed using software package Pyris for Windows, version 3.81.

2.2.2. TGA

TGA curves were collected using TGA 7 instrument, Perkin-Elmer (USA). Approximately, 5 mg of sample was placed in open platinum pan and heated from ambient temperature to 700 °C at heating rate of 10 °C min^{-1} under flow of nitrogen (35 ml min^{-1}).

2.3. Synthesis

2.3.1. Materials

Dialysis was performed with Visking Dialysis Tubing 45/55 cm (Serva, Germany) with a molecular cut-off 8000−15,000. For thin layer chromatography, silica gel sheets Kieselgel 60 F_{254} (Merck, Germany) were used. Solvent systems were cyclohexane/ethyl acetate

1:1, toluene/ethyl acetate 3:7 and chloroform. For spot detection iodine vapour was used. Estradiol-3-benzoate was purchased from Sigma (USA) and benzotriazole and triphosgene from Aldrich (USA). All solvents were of analytical grade purity and dry.

2.3.2. 1-Benzotriazole carboxylic acid chloride (BtcCl, **1**)

The compound **1** was prepared from benzotriazole and triphosgene as described previously (Kalčić et al., 2003).

2.3.3. Estradiol-3-benzoate-17-(benzotriazole-1-carboxylate) (**2**)

A solution of 0.181 g (0.001 mol) BtcCl (**1**) in 40 ml toluene was added drop-wise to a solution of 0.376 g (0.001 mol) estradiol-3-benzoate (EB) and 0.101 g (0.001 mol) triethylamine (TEA) in 20 ml dry toluene. The reaction mixture was stirred for 72 h at room temperature. During that period three portions of 0.181 g BtcCl and 0.101 g TEA in 30 ml toluene were added to the reaction mixture in 24 h intervals. The precipitated TEA hydrochloride was filtered off and the mother liquor was extracted 5 times with diluted HCl (w = 2%) and several times with water. The organic layer was dried over sodium sulphate, filtered and evaporated to dryness. The oil product **2** crystallized by triturating with petroleum ether. Yield: 0.414 g (79%). m.p. 165−168 °C. IR (KBr): ν_{max} 1759, 1728, 1495, 1450, 1264, 1049, 762, 707 cm^{-1}.

^{1}H NMR (DMSO-d_6), δ (ppm): 8.25 (m, 1H, arom. H 22-25), 8.12 (t, 2H, H-28, 32), 7.80 (m, 1H, arom. H 22-25), 7.75 (t, 1H, H-30), 7.74 (m, 1H, arom. H 22-25), 7.61 (t, 2H, H-29, 31), 7.38 (d, 1H, H-1), 7.02 (dd, 1H, H-2), 6.98 (d, 1H, H-4), 5.12 (t, 1H, H-17), 2.89 (m, 2H, H-6), 2.39 (m, 2H, H-16), 2.38 (m, 1H, H-11), 2.33 (m, 1H, H-9), 2.02 (m, 2H, H-12), 1.94 (m, 2H, H-16), 1.90 (m, 1H, H-7), 1.82 (m, 2H, H-15), 1.59 (m, 2H, H-12), 1.51 (m, 2H, H-15), 1.50 (m, 1H, H-14), 1.50 (m, 1H, H-11), 1.49 (m, 1H, H-8), 1.40 (m, 1H, H-7), 1.03 (t, 3H, H-18). ^{13}C NMR (DMSO-d_6), δ (ppm): 164.20 (C-26), 148.30 (C-3), 148.15 (C-19), 137.77 (C-5), 137.35 (C-10), 133.86 (C-30), 130.68 (C-22), 129.59 (C-27), 129.18 (C-23), 128.95 (C-28, 32), 128.86 (C-29, 31), 126.32 (C-1), 125.44 (C-24), 121.47 (C-4), 119.62 (C-25), 118.83 (C-2), 112.86 (C-20), 87.13 (C-17), 43.23 (C-9), 48.64 (C-14), 42.97 (C-13), 37.72 (C-8), 36.19 (C-12), 28.81 (C-6), 26.87 (C-16), 26.38 (C-7), 25.55 (C-11), 22.70 (C-15), 12.16 (C-18) (atom enumeration is presented in Fig. 1). Elemental analysis for $C_{32}H_{31}N_3O_4$ (521.61) (%): calcd. C 73.69, H 5.99, N 8.06; found: C 73.59, H 5.66, N 8.17.

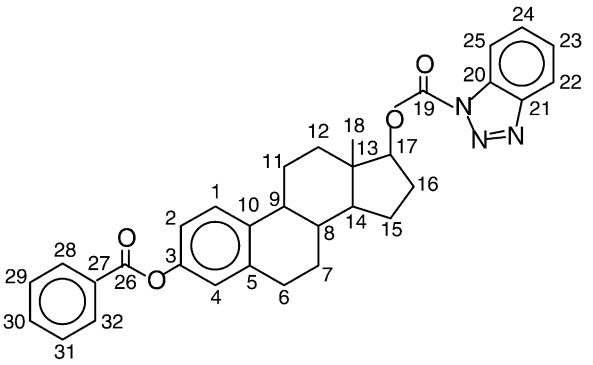


Fig. 1. Atom enumeration of estradiol-3-benzoate-17-(benzotriazole-1-carboxylate) (**2**).

2.3.4. Poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))–poly(α,β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer (PAHA, **3**)

PAHA was synthesized by partial aminolysis of polyssucinimide (PSI) with 2-aminoethanol and subsequently with 1,2-diaminoethane (Zorc et al., 1995). Weight average molecular weight of PSI was 55000 and it was determined by viscosimetric method following Mark–Houwink equation $[\eta] = 1.32 \times 10^{-2}\,M^{0.76}$ (Vlasák et al., 1979). The molar ratio of PSI monomer and 2-aminoethanol was 3:1 and 1,2-diaminoethane was used in excess in order to prevent cross-linking.

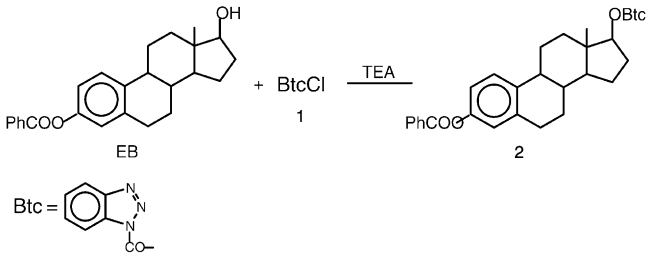
2.3.5. PAHA–estradiol-3-benzoate conjugate (PAHA–EB, **4**)

A solution of 0.198 g (0.0038 mol) compound **2**, 2.687 g (0.0266 mol) TEA and 1.799 g (0.0114 mol) PAHA in 200 ml *N*,*N*′-dimethylformamide (DMF) was stirred at room temperature for 72 h. The solvent was evaporated under reduced pressure and the residue was triturated with acetone. The crude product **4** was filtered off, dissolved in water, dialyzed against several changes of deionized water during 3 days and lyophilized. Yield: 1.052 g (54%). IR (KBr): ν_{max} 3310, 3080, 2941, 1736, 1732, 1652, 1540, 1386, 1265, 1227, 1176, 1150,

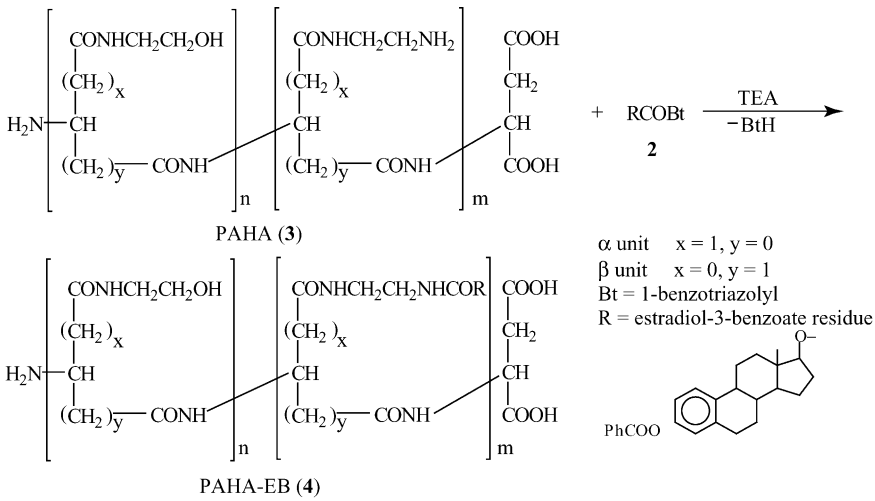
1064, 893, 709, 668 cm^{-1}. The conjugate **4** is freely soluble in water (50 mg/ml).

3. Results and discussion

1-Benzotriazole carboxylic acid chloride (BtcCl, **1**) is an azole derivative useful in preparation of different classes of organic compounds, e.g. esters, amides, ureas, hydroxyureas, hydantoins, carbamates, carbazides, oligopeptides, *N*-hydroxyisocyanate derivatives (see for example: Butula et al., 1983; Zorc et al., 1990; Muskolaj et al., 1997; Butula and Jadrijević-Mladar Takač, 2000). In current investigation it has been used for activation of C-17 hydroxyl group in estrogen-3-benzoate (EB). The compound **1** was prepared from benzotriazole and triphosgene following the recently modified procedure (Kalčić et al., 2003). Reaction of chloride **1** and EB is presented in Scheme 1. Equimolar amount of BtcCl was not sufficient to accomplish reaction so additional amount of the reagent and triethylamine (TEA) was needed. The product of the reaction, estradiol-3-benzoate-17-(benzotriazole-1-carboxylate) (**2**) was characterized by ^{1}H and ^{13}C NMR spectroscopy, IR and elemental analysis (see experimental part and Fig. 1). The proton and carbon chemical shifts were unambiguously assigned by the combined use of one- (^{1}H and APT) and two-dimensional NMR spectra (COSY, TOCSY, HSQC and HMBC). 2D spectra showed the expected correlation peaks characteristic of the structure depicted in Fig. 1. This is especially true for the TOCSY spectrum shown in Fig. 2 where magnetisation is transferred between directly coupled protons in a stepwise fashion identifying different spin systems within the molecule. The HSQC spectra yielded an unambiguous assignment for the protonated carbon atoms while correlation peaks in HMBC spectra



Scheme 1.



Scheme 2.

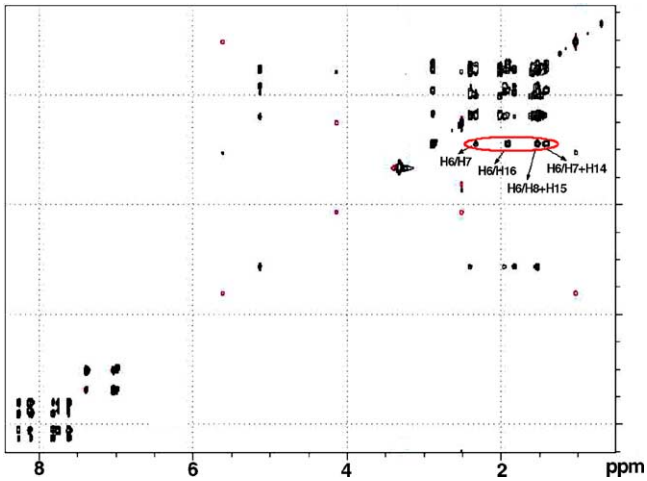


Fig. 2. The TOCSY spectrum of compound **2**. Cross-peaks are labeled indicating the spin system in the steroid part of the molecule.

revealed information about the quaternary carbons and confirmed the assignments of the protonated carbons.

Compound **2** is an active carbamate in which the benzotriazole moiety is readily substituted by stronger nucleophiles (Butula et al., 1977). For our purposes, poly(α,β-(*N*-2-hydroxyethyl-DL-aspartamide))–poly(α,β-(*N*-2-aminoethyl-DL-aspartamide)) copolymer (PAHA) was chosen as polyamino nucleophile. Reaction of **2** and PAHA afforded PAHA–EB conjugate (**4**), in which estradiol moiety is covalently bond

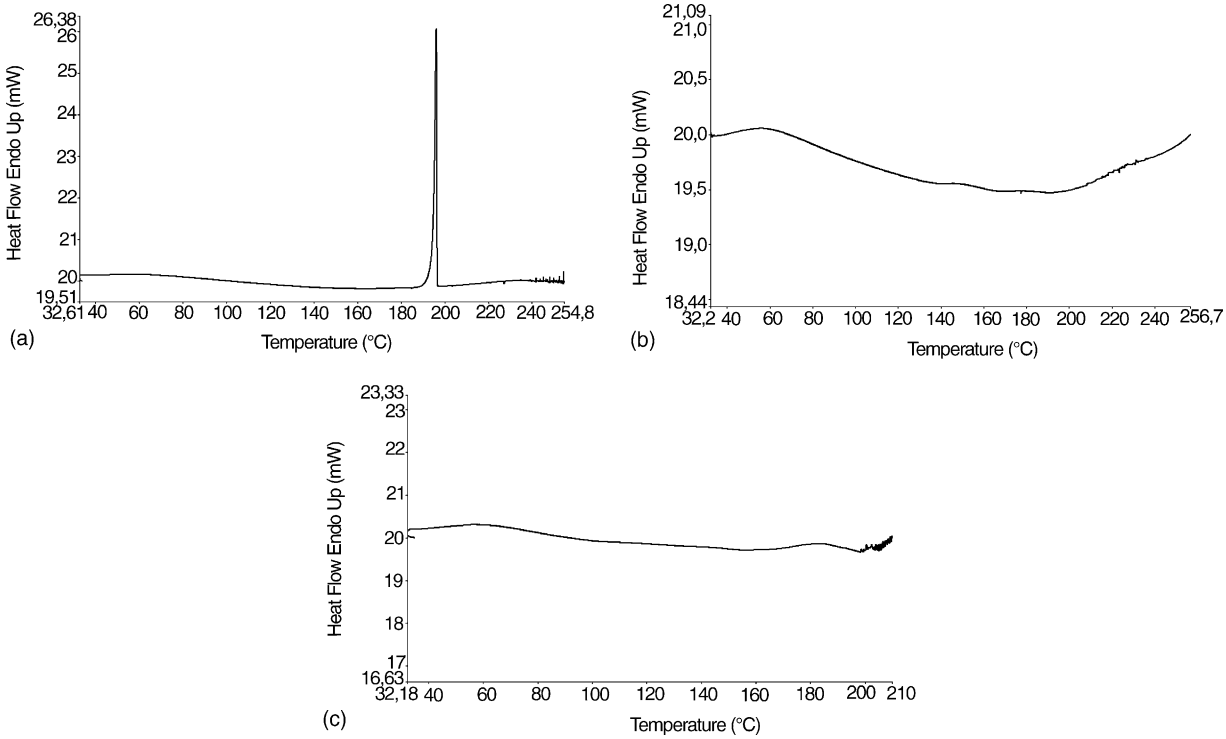


Fig. 3. DSC thermograms of (a) estradiol-3-benzoate, (b) PAHA and (c) PAHA–EB.

to polymeric carrier by carbamate linkage, through non-toxic ethylenediamine spacer Scheme 2.

The reaction was performed in DMF solution, in molar ratio 1:3 (calculated as monomer units), which theoretically enabled substitution of each amino group by estradiol derivative. However, binding of estradiol moiety was not quantitative.

PAHA-EB gave a positive reaction with sulphomolybdic reagent as estradiol-3-benzoate itself (European Pharmacopoeia, 1997). The drug loading in the conjugate was estimated by UV spectroscopy using the calibration curve for EB in ethanol/water 1:1 (v/v) at $\lambda = 233$ nm (apsorption maximum of EB). The estradiol loading was estimated as 29% (m/m). The result was confirmed by hydrolysis of the conjugate in phosphate buffer pH 9.

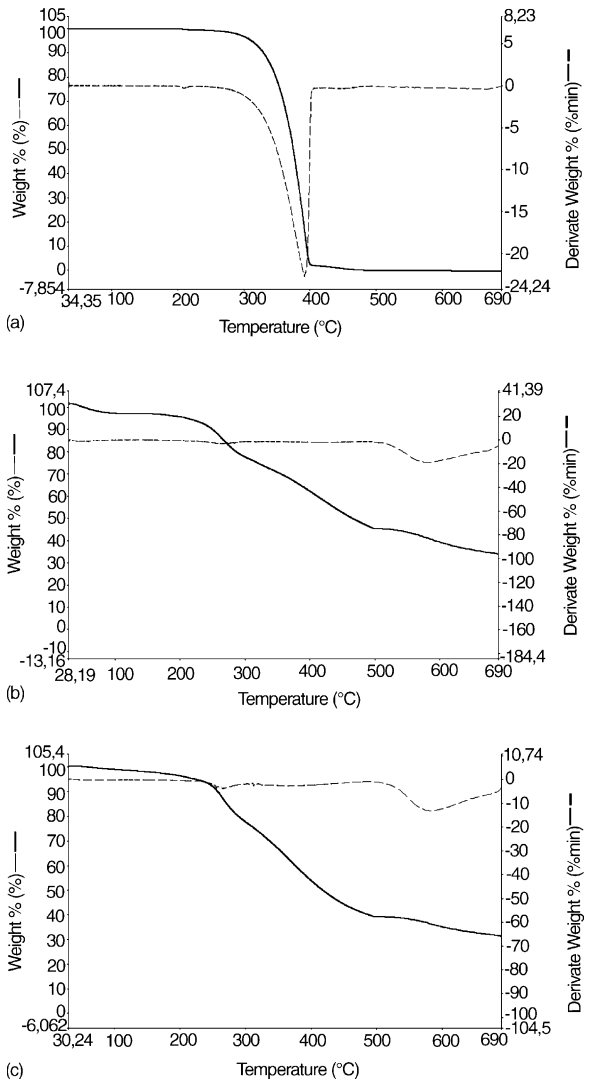


Fig. 4. TGA thermograms of (a) estradiol-3-benzoate, (b) PAHA and (c) PAHA–EB.

EB, PAHA and PAHA-EB are characterized by differential scanning calorimetry and thermogravimetry (Figs. 3 and 4). DSC thermogram of EB exhibits a wide endothermic peak at 56.5 °C ($\Delta H = 42.7\,J\,g^{-1}$) that corresponds to exit of adsorbed moisture, which is also detected as 0.5% of weight loss by TGA. The second endothermic peak observed at 194.8 °C ($\Delta H = 94.2$ °C) corresponds to the melting of EB. DSC thermogram for PAHA shows endothermic peak at 38.2 °C ($\Delta H = 233.9\,J\,g^{-1}$) that represents solvent/moisture loss. The solvent/moisture loss of 4.5% in temperature region from 30.7 to 106.8 °C is confirmed by TGA. DSC thermogram of PAHA–EB exhibits only a wide endothermic peak at 67.1 °C ($\Delta H = 172.8\,J\,g^{-1}$), related to solvent/moisture loss. Endothermic peak that corresponds to melting of EB is absent which clearly indicates that EB is conjugated to PAHA. TGA curve for the conjugate **4** showed continuous weight loss during the heating, with total degradation of the compound above 250 °C.

Attachment of estrogen derivative to the macromolecular carrier would limit its cellular uptake to the endocytic route (Duncan, 1992). One could expect that acidic pH and hydrolytic enzymes within lysosomal compartment of the cell would cause the drug release from the conjugate. Due to enhanced hydrosolubility altered pharmacokinetics could be expected as well.

4. Conclusion

Here described new compounds, estradiol benzotriazole derivative (**2**) and PAHA–estradiol benzoate conjugate (**4**) could be potential estrogen prodrugs with expected differences in solubility and transport behaviour relative to usual steroid esters.

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