Acta Pharm. **61** (2011) 465–472
DOI: 10.2478/v10007-011-0039-x

Short communication

Macromolecular prodrugs. XIII. Hydrosoluble conjugates of 17β-estradiol and estradiol-17β-valerate with polyaspartamide polymer

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Two hydrosoluble conjugates of 17β-estradiol (ED) and estradiol-17β-valerate (EV) with polyaspartamide polymer were prepared and characterized. ED and EV were first chemically modified and bound to poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)] (PAHA), a hydrosoluble polyaspartamide-type copolymer bearing both hydroxyl and amino groups. ED was first converted to 17-hemisuccinate (EDS) and then bound to PAHA. In the resulting conjugate PAHA-EDS, the estradiol moiety was linked to the polymer through a 2-aminoethylhemisuccinamide spacer. On the other hand, EV was first converted to estradiol-17β-valerate-3-(benzotriazole-1-carboxylate), which readily reacted with amino groups in PAHA affording the polymer-drug conjugate PAHA-EV. In the prepared conjugate PAHA-EV, the estradiol moiety was covalently bound to the polyaspartamide backbone by carbamate linkage, through an ethylenediamine spacer. The polymer-drug conjugates were designed and prepared with the aim to increase water-solubility, bioavailability and to improve drug delivery of the lipophilic estrogen hormone.

Keywords: estradiol, polyaspartamide, poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)], polymer-drug conjugate

Accepted November 17, 2011

Polymer-drug conjugates are macromolecular drug carrier systems with active agents attached as side substituents to a polymer backbone using an appropriate spacer. They could be an effective way to prolong drug activity, minimize unfavorable side effects and toxicity, decrease the required dose and increase the solubility of drugs (1). In addition, polymer-drug conjugates have the ability to overpass some mechanisms of drug resistance and the potential to elicit immunostimulatory effects. They can also alter the body distribution of drugs and ensure adequate drug delivery to target cells or tissues. This is

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particularly advantageous in cancer therapy since polymers accumulate in malignant cells more readily than in healthy tissues, thus precluding undesirable side effects generated by free drugs (2).

Polymers of polyaminoacid type are frequently used in polymer-drug conjugates because of their protein-like nature and biodegradability. Polymers of controlled molecular mass and molecular mass distribution can be prepared by chemical or biosynthetic methods. Polyaminoacid polymers with various side functional groups suitable for modification and conjugation with active molecules are available (3). Among polyaminoacids, polyaspartamide derivatives have been of special interest due to their excellent solubility, bioavailability, biocompatibility and favorable reactivity (1). Polyaspartamide conjugates with numerous drugs have been reported by our (4–6) and other research groups (see, for example, refs. 7, 8).

Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)] (PAHA) is a copolymer of polyaspartamide type that has been successfully applied in the synthesis of several macromolecular prodrugs, including 17β-estradiol-3-benzoate (9), diclofenac, fenoprofen (10) and broxuridine (11).

In this paper, we report the synthesis of two hydrosoluble PAHA conjugates of 17β--estradiol (ED) and estradiol-17β-valerate (EV), prepared with the aim to increase water-solubility, bioavailability and improve drug delivery of this estrogen hormone.

In addition to serving as water-soluble prodrugs of estrogen, such conjugates have potential uses as carriers for antitumor drugs. Polymer-drug conjugates with chemically bonded steroid hormones have been explored as a means of targeting the active molecule to tumor tissue due to the presence of tumor-associated receptors for steroid hormones (12). It was thus proposed that estrogens chemically coupled to an active molecule *via* an appropriate spacer could, upon binding to nuclear steroid receptors, direct the active molecule into the nucleus of tumor cells (13).

EXPERIMENTAL

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 (Bruker, USA) operating at 500.13 MHz for ^{1}H and equipped with a 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 32000 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 a 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 a 2 K × 1 K transformed matrix. The sweep width was 6600 Hz in f2 di-

mension 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. 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).

Dialysis was performed with Visking Dialysis Tubing 18/22 (Serva, Germany). For thin layer chromatography, silica gel sheets Kieselgel 60 F_{254} (Merck, Germany) were used. Solvent systems were cyclohexane/ethyl acetate 1:1 and 1:3, or chloroform. For spot detection, iodine vapor and short-wave UV light were used. Column chromatography was performed on silica gel 0.063-0.200 mm (Kemika, Croatia), with chloroform as eluent. Estradiol, estradiol-17β-valerate, benzotriazole, succinic anhydride, triphosgene and 1-ethyl--3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich (USA). All solvents were of analytical grade and were dried before use.

1-Benzotriazole carboxylic acid chloride (BtcCl, **1**) was prepared from benzotriazole and triphosgene following the procedure published previously (14).

Syntheses

*Estradiol-17β-hemisuccinate (EDS, **2**).* – ES was prepared according to a modified procedure (15). A solution of 0.327 g (0.0012 mol) estradiol and 0.120 g (0.0012 mol) succinic anhydride in 15 mL pyridine was refluxed for 14 h. An additional amount of 1.200 g (0.012 mol) succinic anhydride was added in portions of 0.120 g approximately each hour. The reaction mixture was poured into 600 mL water and the suspension was extracted three times with ether. Organic layers were combined and washed with diluted HCl solution (w = 2 %) and water until neutral, dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 15 mL methanol and treated with a solution of 0.332 (0.0024 mol) potassium carbonate in 5 mL water and 15 mL methanol. The reaction mixture was stirred for 24 h and evaporated. The crude product was mixed with 30 mL water and extracted three times with ether. Organic layers were combined, dried over anhydrous sodium sulfate and evaporated under reduced pressure.

*Estradiol-17β-valerate-3-(benzotriazole-1-carboxylate) (EVB, **3**).* – A solution of 0.036 g (0.0002 mol) BtcCl (**1**) in 5 mL toluene was added dropwise to a solution of 0.071 g (0.0002 mol) EV and 0.020 g (0.0002 mol) triethylamine (TEA) in 20 mL dry toluene. The reaction mixture was stirred for 1 h at room temperature and evaporated under reduced pressure. The analytically pure product **3** was obtained after column chromatography (mobile phase: chloroform).

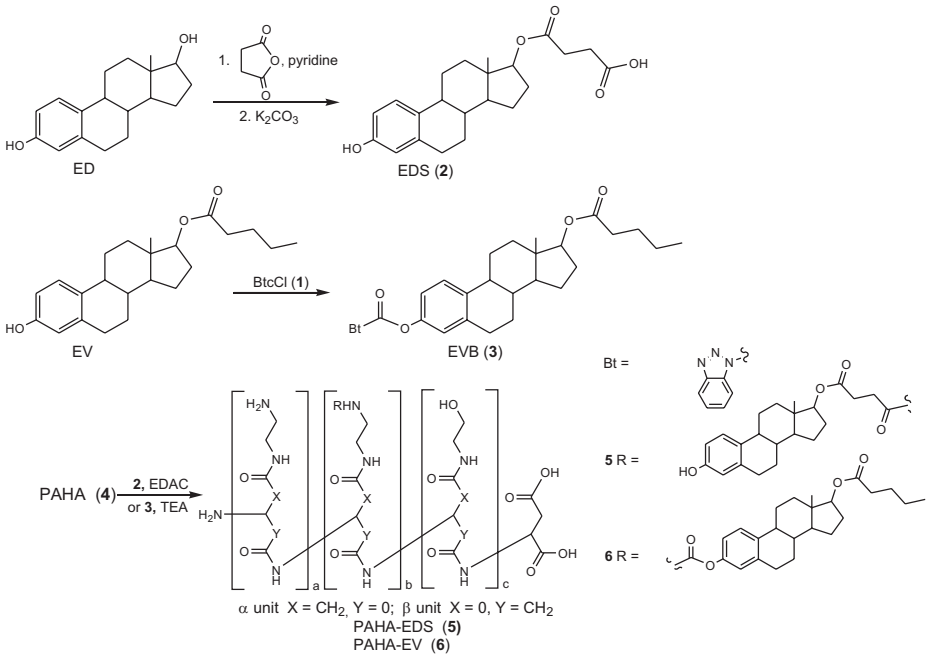
*Poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-aminoethyl-DL-aspartamide)] copolymer (PAHA, **4**).* – PAHA was synthesized by partial aminolysis of polysuccinimide (PSI) with 2-aminoethanol and subsequently with ethylenediamine following a previously described procedure (11). Average molecular mass of PSI was determined by the viscosimetric method following the Mark-Houwink equation $[\eta] = 1.32 \times 10^{-2}\ M^{0.76}$ (M_r = 55000). The molar ratio of PSI monomer to 2-aminoethanol was 3:2 and ethylenediamine was used in excess in order to prevent cross-linking.

*PAHA-estradiol-3-benzoate-17β-hemisuccinate conjugate (PAHA-EDS, **5**).* – PAHA-EDS conjugate was prepared by the coupling reaction of PAHA (0.095 g, 0.006 mol) and EDS (**2**) (0.075 g, 0.002 mol) in the presence of EDAC (0.959 g, 0.005 mol) in a DMF/water/acetone mixture (25:18:8, *V/V/V*) at pH 5. Reaction mixture was stirred for 72 h. Product **5** was purified by dialysis against water, followed by freeze drying. The purity of the product was examined by TLC. Drug loading was determined by UV spectrophotometry at 280 nm from the calibration curve of estradiol (7 %).

*PAHA-estradiol-17β-valerate conjugate (PAHA-EV, **6**).* – A solution of 0.108 g (0.0002 mol) of compound **3**, 0.283 g (0.0028 mol) TEA and 0.094 g (0.0006 mol) PAHA in 20 mL DMF was stirred for 72 h at room temperature. The solvent was evaporated under reduced pressure and the residue was triturated with acetone. Product **6** was filtered off, dissolved in water, dialyzed for 3 days and freeze-dried. Drug loading was determined by UV spectrophotometry at 280 nm from the calibration curve of estradiol (12 %).

RESULTS AND DISCUSSION

Two hydrosoluble conjugates of 17β-estradiol (ED) and its ester estradiol-17β-valerate (EV) with poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)]-poly[α,β-(*N*-2-aminoethyl--DL-aspartamide)] copolymer (PAHA, **4**) were prepared and characterized. PAHA was



Scheme 1

prepared following our synthetic procedure by partial aminolysis of polyssucinimide with 2-aminoethanol in a ratio 3:2 (calculated as PSI monomer unit) and followed by aminolysis with an excess of ethylenediamine in order to prevent cross-linking (11). Prior to binding, ED and EV were chemically modified. ED was first converted to 17-hemisuccinate (EDS, **2**), a derivative bearing a free carboxylic group, which enabled binding to PAHA by an amide bond. EDS was prepared in the reaction of ED with an excess of succinic acid anhydride in pyridine (Scheme 1). In that reaction, both hydroxyl groups of ED reacted with anhydride forming a diester. By addition of potassium, the carbonate ester bond at the phenolic group was cleaved. The coupling of EDS to PAHA was performed in an EDS/PAHA molar ratio 1:3, in the presence of a water-soluble carbodiimide EDAC. In the resulting conjugate PAHA-EDS (**5**), the estrogen moiety was linked to the succinate spacer by an easily cleavable ester bond.

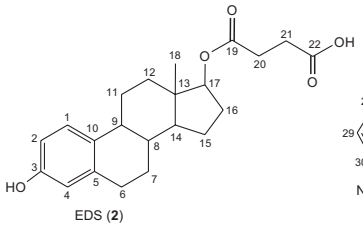
EV was first converted to estradiol-17β-valerate-3-(benzotriazole-1-carboxylate) (EVB, **3**). Compound **3** was prepared from EV and 1-benzotriazole carboxylic acid chloride (BtcCl, **1**), a versatile azole derivative useful in preparation of different classes of organic compounds. Compound **3** is a reactive carbamate, which readily reacted with amino groups in PAHA affording the polymer-drug conjugate PAHA-EV (**6**). In conjugate **6**, the estradiol moiety was covalently bound to the polyaspartamide backbone by carbamate linkage, through an ethylenediamine spacer. Synthesis of conjugate **6** proceeded in DMF at room temperature, in the presence of TEA. The molar ratio of the reactants was 1:3 (calculated as monomer units), which theoretically enabled substitution of each amino group by the estradiol derivative. However, binding of the estradiol moiety was not quantitative and the final conjugate **6** contained 12 % (*m/m*) of estrogen.

The synthesized compounds were characterized by ^{1}H, ^{13}C NMR, UV and IR spectroscopy (Tables I and II). The proton and carbon chemical shifts were assigned by the combined use of one- (^{1}H and APT) and two-dimensional NMR spectra (COSY, TOCSY, HSQC and HMBC).

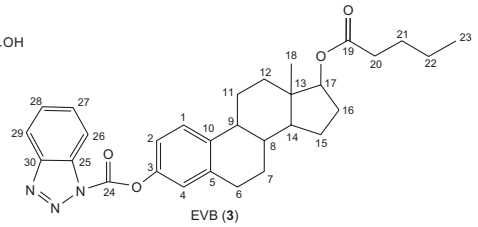
Table I. Physico-chemical data for compounds ***2****,* ***3****,* ***5****,* ***6***

Compd.	Yield (%)	M.p. (°C)	Drug loading (%)	IR(KBr): ν_{max} (cm^{-1})	Elemental analysis calcd./found		
					C	H	N
2	58	167–169	–	3425, 3321, 2931, 1725, 1402, 1220, 1170, 1001, 874	70.94 70.78	7.58 7.66	– –
3	45	165–166	–	2929, 2869, 1761, 1731, 1609, 1488, 1453, 1393, 1248, 1025, 749	71.83 71.54	7.03 6.96	8.38 8.70
5	62	–	7	3308, 1652, 1539, 1435, 1287, 1251, 1180, 1062, 668	–		
6	53	–	12	3379, 3070, 2929, 1653, 1542, 1438, 1388, 1279, 1232, 1175, 1059, 1064, 668	–		

*Table II. ^1H and ^{13}C NMR spectra of estradiol derivatives **2** and **3***



EDS (**2**)



EVB (**3**)

Compd.	**2**		**3**	
Atom	^1H δ (ppm)	^{13}C δ (ppm)	^1H δ (ppm)	^{13}C δ (ppm)
1	7.03	125.95	7.44	126.62
2	6.50	112.64	7.27	118.43
3	–	154.85	–	147.60
4	4.43	114.83	7.22	121.09
5	–	136.97	–	138.59
6	2.71	28.99	2.88	28.86
7	1.79, 1.26	26.74	1.67, 1.37	26.33
8	1.30	38.19	1.51	37.64
9	2.08	43.14	2.29	43.34
10	–	130.04	–	137.96
11	2.22, 1.30	25.76	2.36, 1.43	25.56
12	1.76, 1.30	36.32	1.76, 1.40	36.35
13	–	42.56	–	42.43
14	1.26	48.96	1.35	48.98
15	1.67, 1.35	22.69	1.71, 1.37	22.07
16	2.08, 1.49	26.98	2.11, 1.51	27.08
17	4.62	81.84	4.66	81.52
18	0.77	11.77	0.82	11.83
19	–	171.75	–	172.08
20	2.50	28.94, 28.73	2.31	33.39
21	2.50	–	1.53	26.64
22	–	173.31	1.31	21.50
23	–	–	0.88	13.49
24	–	–	–	160.55
25	–	–	–	113.07
26	–	–	8.29	119.73
27	–	–	7.63	125.71
28	–	–	7.81	130.24
29	–	–	8.12	130.96
30	–	–	–	133.68

Drug loading in the conjugates was estimated by UV spectroscopy using the calibration curve for ED in ethanol/water 1:1 (*V/V*) at λ = 280 nm (ED absorption maximum). Drug loading in conjugates **5** and **6** was 7 and 12 % (*m/m*), respectively. Both conjugates were freely soluble in water. Preliminary hydrolysis studies showed that the drug could be released from macromolecular prodrugs after chemical hydrolysis in a wide pH range, but detailed kinetic studies and evaluation of potential pharmaceutical uses still remain to be done.

CONCLUSIONS

Estrogen hormone 17β-estradiol and its ester with valeric acid were linked to the polyaspartamide-type polymer PAHA, providing hydrosoluble polymer-drug conjugates PAHA-EDS (**5**) and PAHA-EV (**6**). The conjugates differ in the type of spacer and in drug loading. In conjugate **5**, estradiol was linked to the spacer and then to the polymer through the C-17 hydroxy group, whereas through the C-3 phenol group in conjugate **6**. The synthesized conjugates are potential estrogen prodrugs.

Acknowledgements. – Support for this study was provided by the Ministry of Science, Education and Sports of the Republic of Croatia (Projects No. 006-0000000-3216 and 119-1191342-1083).

REFERENCES

1. F. M. Veronese and M. Morpurgo, Bioconjugation in pharmaceutical chemistry, *Farmaco* **54** (1999) 497–516; DOI: 10.1016/S0014-827X(99)00066-X.
2. F. Greco and M. J. Vicent, Combination therapy: Opportunities and challenges for polymer-drug conjugates as anticancer nanomedicines, *Adv. Drug Deliver. Rev.* **61** (2009) 1203–1213; DOI: 10.1016/j.addr.2009.05.006.
3. G. Pitarresi, F. Saiano, G. Cavallaro, D. Mandracchia and F. S. Palumbo, A new biodegradable and biocompatible hydrogel with polyaminoacid structure, *Int. J. Pharm.* **335** (2007) 130–137; DOI: 10.1016/j.ijpharm.2006.11.012.
4. B. Zorc, M. Ljubić, S. Antolić, J. Filipović-Grčić, D. Maysinger, T. Alebić-Kolbah and I. Jalšenjak, Macromolecular prodrugs. II. Esters of L-dopa and α-methyldopa, *Int. J. Pharm.* **99** (1993) 135–143.
5. M. Lovrek, B. Zorc, B. Boneschans and I. Butula, Macromolecular prodrugs. VIII. Polymer-gemfibrozil conjugates, *Int. J. Pharm.* **200** (2000) 59–66.
6. M. Zovko, B. Zorc, M. Lovrek and B. Boneschans, Macromolecular prodrugs. IX. Synthesis of polymer-fenoprofen conjugates, *Int. J. Pharm.* **228** (2001) 129–138.
7. G. Cavallaro, L. Maniscalco, M. Campisi, D. Schillaci and G. Giammona, Synthesis, characterization and in vitro cytotoxicity studies of a macromolecular conjugate of paclitaxel bearing oxytocin as targeting moiety, *Eur. J. Pharm. Biopharm.* **66** (2007) 182–192; DOI: 10.1016/j.ejpb.2006.10.013.
8. G. Cavallaro, S. Scirè, M. Licciardi, M. Ogris, E. Wagner and G. Giammona, Polyhydroxyethylaspartamide-spermine copolymers: Efficient vectors for gene delivery, *J. Control. Release* **131** (2008) 54–63; DOI: 10.1016/j.jconrel.2008.07.001.

9. M. Zovko, B. Zorc, P. Novak, P. Tepeš, B. Cetina-Čižmek and M. Horvat, Macromolecular prodrugs. XI. Synthesis and characterization of polymer-estradiol conjugate, *Int. J. Pharm.* **285** (2004) 35–41; DOI: 10.1016/j.ijpharm.2004.07.013.
10. M. Barbarić, M. Kralj, M. Marjanović, I. Husnjak, K. Pavelić, J. Filipović-Grčić, D. Zorc and B. Zorc, Synthesis and in vitro antitumor effect of diclofenac and fenoprofen thiolated and non-thiolated polyaspartamide-drug conjugates, *Eur. J. Med. Chem.* **42** (2007) 20–29; DOI: 10.1016/j.ejmech.2006.08.009.
11. B. Zorc, D. Maysinger, I. Kalčić and I. Butula, Macromolecular prodrugs. V. Polymer-broxuridine conjugates, *Int. J. Pharm.* **123** (1995) 65–70; DOI: 10.1016/0378-5173(95)00040-P.
12. A. Agarwal, S. Saraf, A. Asthana, U. Gupta, V. Gajbhiye and N. K. Jain, Ligand based dendritic systems for tumor targeting, *Int. J. Pharm.* **350** (2008) 3–13; DOI: 10.1016/j.ijpharm.2007.09.024.
13. G. M. Dubowchik and M. A. Walker, Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer, *Pharmacol. Therapeut.* **83** (1999) 67–123; DOI: 10.1016/S0163-7258(99)00018-2.
14. I. Kalčić, M. Zovko, M. Jadrijević-Mladar Takač, B. Zorc and I. Butula, Synthesis and reactions of some azole carboxylic acid derivatives, *Croat. Chem. Acta* **76** (2003) 217-228.
15. T. O. Yellin, Estradiol 17β-hemisuccinate: an improved procedure, *J. Lipid Res.* **13** (1972) 554–555.

S A Ž E T A K

Makromolekulski prolijekovi. XIII. Vodotopljivi konjugati 17β-estradiola i estradiol-17β-valerata s poliaspartamidnim polimerom

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U radu je opisana priprava i karakterizacija dvaju vodotopljivih polimer-lijek konjugata 17β-estradiola (ED) i estradiol-17β-valerata (EV) s poliaspartamidnim polimerom. ED i EV su prvo kemijski modificirani i vezani na poli[α,β-(*N*-2-hidroksietil-DL-aspartamid)]-poli[α,β-(*N*-2-aminoetil-DL-aspartamid)] (PAHA), vodotopljivi poliaspartamidni kopolimer s hidroksi i amino skupinama. ED je prvo preveden u 17-hemisukcinat (EDS), a zatim vezan na PAHA. U nastalom konjugatu PAHA-EDS estradiolska komponenta vezana je na polimer preko 2-aminoetilhemisukcinatne razmaknice. S druge strane, EV je prvo preveden u estradiol-17β-valerat-3-(benzotriazol-1-karboksilat), koji reagira s amino skupinama u PAHA dajući polimer-lijek konjugat PAHA-EV. U tom je konjugatu estradiolska komponenta kovalentno vezana na poliaspartamidnu okosnicu karbamatnom vezom, preko etilenediaminske razmaknice. Polimer-lijek konjugati estradiola dizajnirani su i pripravljeni sa ciljem da se poveća topljivost i bioraspoloživost te isporuka tog lipofilnog estrogenog hormona.

Ključne riječi: estradiol, poliaspartamid, poli[α,β-(*N*-2-hidroksietil-DL-aspartamid)]-poli[α,β-(*N*-2-aminoetil-DL-aspartamid)], polimer-lijek konjugat

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