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Synthesis of dehydroepiandrosterone analogues modified with phosphatidic acid moiety

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ABSTRACT

Dehydroepiandrosterone (DHEA) and its metabolite 7α-OH DHEA have many diverse physiological, biological and biochemical effects encompassing various cell types, tissues and organs. In *in vitro* studies, DHEA analogues have myriad biological actions, but *in vivo*, especially in oral administration, DHEA produces far more limited clinical effects. One of the possible solutions of this problem is conversion of DHEA to active analogues and/or its transformation into prodrug form. In this article, the studies on the conversion of DHEA and 7α-OH DHEA into their phosphatides by the phosphodiester approach are described. In this esterification, *N*,*N*-dicyclohexylcarbodiimide (DCC) was the most efficient coupling agent as well as *p*-toluenesulphonyl chloride (TsCl).

1. Introduction

Dehydroepiandrosterone (DHEA), together with its sulphate ester DHEA-S, is a naturally occurring steroid that is mainly produced in the adrenal gland and in different tissues. Moreover, DHEA is synthesised *de novo* in the brain, where it plays an important role as a neuroactive molecule [1–3]. Dehydroepiandrosterone is an essential intermediate in the biosynthetic conversion of cholesterol to the sex hormones such as testosterone or oestradiol [4,5].

The wide range of DHEA activity has caused that it to be described as a 'super hormone' [6]. It helps with obesity, decreases blood cholesterol, decreases blood glucose level, enhances the immune system, reduces and prevents tumours, enhances memory function, prevents or slows the progression of Alzheimer's and Parkinson's diseases, boosts libido and helps in the treatment of systemic lupus erythematosus [6–9].

It is also worth notice that DHEA has been extensively studied with great success in rodents, but with only equivocal results in man. Unfortunately, in some cases, oral DHEA administration leads to limited clinical effects [4,10,11]. This may be attributed to poor bioavailability and inefficient conversion to active metabolites, and ready conversion of DHEA to sex hormones, properties which result in increase of undesirable androgenic and oestrogenic side effects, rather than the anticipated clinical benefit [4,11]. In this manner, research on DHEA was directed into its analogues, especially oxidised metabolites, such 7-OH DHEA.

7α-Hydroxy-DHEA is a metabolite of DHEA, which is a natural substance produced in the adrenal gland, gonads and brain. Unlike DHEA, it is not easily converted into the sex hormones, but it does share many properties and physiological actions of DHEA. Moreover, the described immune-stimulating effects of DHEA may be caused due to the conversion of DHEA into 7α-hydroxy-DHEA. The studies suggested that 7α-OH DHEA acts as an anti-glucocorticoid that can block the glucocorticoid-induced immunosuppression [11,12]. If so, the local balance between DHEA metabolites and endogenous glucocorticoids may be disturbed in inflammatory diseases [11–14].

Another way to enhance bioavailability of DHEA and its derivatives from the digestive system could be their transformation into reactive intermediates, which can be further metabolised to active species. Carrier-linked prodrugs are lipidic modifications of compounds, which contain two distinct parts: a drug covalently bound to a lipid moiety, that is, a fatty acid, a glyceride or a phospholipid [15]. Noteworthy are phospholipid modifications of drugs [15]. This kind of compound functionalisation has been used for modulation of lipophilicity [16,17] and to prevent degradation [17]; however, above all, phosphatidylated molecules are characterised by enhanced affinity towards cell membranes by penetrating the lipid bilayers, then liberating the active drug on the inner side of the bilayers [15,18–20]. Moreover, phosphatides as prodrugs exhibit much greater activity then parent compounds [18–20].

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As a part of our studies on phospholipids, we decided to synthesise DHEA and 7α-OH DHEA modified with phosphatidic acid (PA) residue as a potential prodrug. It is known that dehydroepiandrosterone 1,2-dipalmitoyl-*sn*-glycero-3-phosphatide (DPP-DHEA) is a more effective inhibitor of glucose-6-phosphate dehydrogenase than is the free form of DHEA [21]. The synthesis of DPP-DHEA was described by Ortel and Benes [21] and by Williams and Boehm [22] and both groups used commercial PA. Our idea is based on the application of phosphatidylcholine (PC, lecithin) isolated from the egg yolk of Lohmann Brown hens' as a source of natural PA. Here, the synthesis of 1,2-diacyl-*sn*-glycero-3-phospho-DHEA analogues are described.

2. Experimental

2.1. General methods

All reagents were purchased from Sigma–Aldrich Chemical Co. All solvents and reagents were of analytical grade. Solvents were dried and distilled prior to use, according to standard protocols. *sn*-Glycero-3-phosphocholine (GPC) was purchased from Bachem, phospholipase D from *Streptomyces chromofuscus* was purchased from Aldrich. All solvents for liquid chromatography were freshly opened bottles of Merck LiChrosolv® Reag. obtained from Merck.

The Lohmann Brown hens' eggs were a gift from Tronina Factory. PC was isolated as a crude phospholipid fraction by extraction according to Palacios and Wang [23] and purified by column chromatography on a silica gel (eluent: $CHCl_3$:methyl alcohol (MeOH):H_2O from 65:25:0 to 65:25:4, v/v/v).

Electrospray ionisation-mass spectrometry (ESI-MS) were measured on a Bruker micrOTOF-Q. All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance II 600 MHz. Chemical shifts (^{1}H and ^{13}C) (δ) are given in parts per million (ppm) downfield from tetramethylsilane (TMS) as the internal standard. In ^{31}P NMR, chemical shifts were referenced to 85% H_3PO_4 as an external standard. Coupling constant (J) values are in Hz. Unambiguous assignments for all protons and carbons were made through homo- and heteronuclear correlation (^{1}H–^{1}H Correlation Spectroscopy (COSY), ^{1}H–^{13}C Heteronuclear Multiple Quantum Coherence (HMQC) and ^{1}H–^{13}C Heteronuclear Multiple Bond Correlation (HMBC)) of the carbon skeleton.

Thin-layer chromatography (TLC) analyses were carried out on precoated metal plates (0.2-mm silica gel with fluorescent UV254) purchased from Merck. After elution, the plates were developed using the Dittmer reagent or the 0.05% primuline solution (acetone:water, 8:2, v/v) and spots were detected under an ultraviolet (UV) lamp (λ = 365 nm). Silica gel (0.040–0.063 mm, 230–400 mesh ASTM) columns were run under gravity.

DOWEX 50W X8 (pyridinium form) resin was prepared by washing DOWEX 50W X8 (H^+ form) with 50% aqueous pyridine overnight. After this time, resin was washed with water, 50% aqueous MeOH, MeOH and then $CHCl_3$:MeOH:pyridine:H_2O (3:3:1:1, v/v/v/v) [24]. DOWEX 50W X8 (NH_4^+ form) was prepared by the above procedure with using 10% of ammonia solution in water.

2.2. High-performance liquid chromatography analysis

The reactions' course and compounds' purity was controlled by high-performance liquid chromatography (HPLC). As apparatus, an Ultimate 3000 from DIONEX with autosampler and ESA Corona™ Charged Aerosol Detector from ESA Biosciences was used. The detector operated with nitrogen as a nebulising gas and at a range of 100 pA. The Betasil DIOL 5-μm column (Thermo, 150 mm × 2.1 mm) was used. The gradient had a constant flow 0.6 ml min^{-1}, with solvent A = hexane, solvent B = 2-propanol, solvent C = 1% HCOOH, 0.1% TEA in water. Gradient timetable (%A:%B:%C, v/v/v): at 0 min, 40:52:8; at 3 min 40:52:8; at 6 min, 40:50:10; at 11 min, 40:50:10; at 16 min, 37:50:13; at 25 min, 37:50:13; at 25.5 min, 40:52:8 and at 35 min. 40:52:8.

2.3. Analysis of fatty acids composition

Fatty acids composition of appropriate compounds was analysed according to Marinetti [25]. The composition of fatty acids was determined by gas chromatography (GC). The gas chromatograph was an Agilent Technologies 6890N using capillary column TR-FAME (Thermo, 30 m × 0.25 mm × 0.25 μm). Analysis conditions: 140 °C for 3 min, increase temperature 5 °C min^{-1} to 240 °C, increase temperature 30 °C min^{-1} to 260 °C, 260 °C for 2 min.

The composition of fatty acids of lecithin isolated from Lohmann Brown hens' egg yolk was: palmitic acid (16:0), 30%; palmitoleic acid (16:1), 1%; stearic acid (18:0), 18%; oleic acid (18:1), 29%; linoleic acid (18:2), 17%; arachidonic acid (20:4), 3% and docosahexaenoic acid (22:6), 2%.

2.4. Microorganism

Fusarium culmorum was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław (Wrocław, Poland). The microorganism was maintained on Sabouraud 4% dextrose–agar slopes and freshly subcultured before use in the transformation experiments.

2.5. Microbial synthesis of 7α-OH DHEA (**4**)

Aliquots (5 ml) of cell suspension from the seed flask were transferred to seven Erlenmeyer flasks (250 ml), each containing 100 ml of medium consisting of glucose 30 g l^{-1} and peptobac 10 g l^{-1} and incubated for 72 h at 20 °C in a rotary shaker. After this period, 300 mg of DHEA, dissolved in 7 ml of acetone, was evenly distributed between the flasks and incubated further for 24 h, after which the metabolites were extracted from the broth.

The fungal mycelium was separated from the broth by filtration *in vacuo*. Following completion, the mycelium was rinsed with $CHCl_3$ (300 ml). The mycelial broth was then extracted thrice with chloroform (700 ml). The organic extract was dried over anhydrous magnesium sulphate and the solvent was evaporated. The mixture of products (400 mg) was analysed by thin-layer chromatography (TLC) in hexane:acetone (2:1, v/v), and purified by silica-gel chromatography using the same solvents as eluents. This procedure provided 7α-hydroxy-DHEA (264 mg, yield 88%) [26].

2.5.1. 7α-Hydroxy-dehydroepiandrosterone (**4**)

White solid, TLC R_f: 0.14 (hexane:acetone, 2:1, v/v).

ESI-MS m/z: 305.4 $[M+H]^+$.

^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.89 (s, 3H, CH_3-18), 1.02 (s, 3H, CH_3-19), 3.59 (tdd, J = 11.2, 5.0, 4.3 Hz, 1H, H-3), 3.96–4.01 (m, 1H, H-7), 5.65 (dd, J = 5.3, 1.9 Hz, 1H, H-6).

^{13}C NMR (151 MHz, $CDCl_3$) δ: 13.30 (C-18), 18.29 (C-19), 20.10 (C-11), 21.93 (C-15), 31.08 (C-12), 31.31 (C-2), 35.80 (C-1), 36.97 (C-16), 37.22 (C-8), 37.54 (C-10), 41.95 (C-4), 42.65 (C-9), 44.97 (C-14), 47.12 (C-13), 64.31 (C-7), 71.21 (C-3), 123.58 (C-6), 146.60 (C-5), 220.0 (C-17).

2.6. Preparation of sn-glycero-3-phosphocholine × cadmium chloride (GPC × $CdCl_2$) complex

The cadmium chloride complex of GPC was prepared according to the procedure described by Patel et al. [27]. The free GPC (7.71 g, 30 mmol) was dissolved in methanol (50 ml) and added slowly to a solution of cadmium chloride hemi(pentahydride) $CdCl_2 \times 2½H_2O$

(6.85 g, 30 mmol) in water (20 ml). The suspension was mixed in 0 °C for 4 h. The precipitate was filtered off and washed with methanol (20 ml). The precipitate of GPC × $CdCl_2$ was lyophilised from distilled water to give a white powder, which was dried for 24 h in a drying pistol under vacuum over P_2O_5 at the boiling temperature of acetone.

2.7. Synthesis of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

Palmitic acid was dried by repeated co-evaporation with mixture of anhydrous CH_2Cl_2:benzene (1:1, v/v). The complex of GPC × $CdCl_2$ was suspended in the solution of palmitic acid (1.03 g, 4 mmol) in dry CH_2Cl_2 (30 ml) containing 4-(dimethyl-amino)pyridine (244 mg, 2 mmol) and finally, *N*,*N*′-dicyclohexylcarbodiimide (DCC) (865 mg, 4.2 mmol) in a solution of CH_2Cl_2 (10 ml) was added. The suspension was stirred at room temperature under a nitrogen atmosphere. The progress of the reaction was monitored by HPLC and by TLC ($CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v). Lipid spots were detected under a UV lamp after spraying a 0.05% primuline solution (acetone:water, 8:2, v/v).

After 16 h, the precipitate was filtered off and DOWEX 50W X8 (H^+ form) was added to remove the cadmium chloride and 4-(dimethyl-amino)pyridine. The solution was stirred for 30 min, the ion-exchange resin was filtered off and the solvent was evaporated *in vacuo*. The crude product was purified on a silica-gel column (eluent: $CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v). The corresponding fractions (checked on TLC with the primuline test) were collected and evaporated to give appropriate 1,2-diacyl-*sn*-glycero-3-phosphocholines (610 mg, yield 83%). Purity (>98%) was confirmed by HPLC.

2.7.1. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (**1a**)

White solid, TLC R_f: 0.42 ($CHCl_3$:MeOH:H_2O, 65:25:4, v/v/v), HPLC R_t = 15.9 min.

ESI-MS *m*/*z*: 734.6 $[M+H]^+$.

^{1}H NMR (300 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 6.6 Hz, 7H, 2× CH_3-16), 1.27 (s, 48H, 2 × 12 –CH_2–), 1.54–1.69 (m, 4H, 2× CH_2-3), 2.26–2.38 (m, 4H, 2× CH_2-COO), 3.25 (s, 9H, $N(CH_3)_3$), 3.62–3.70 (m, 2H, CH_2-β), 4.03 (dd, *J* = 7.0, 5.6 Hz, 2H, CH_2-3′), 4.16 (dd, *J* = 12.0, 6.9 Hz, 1H, one of H-1′), 4.24–4.36 (m, 2H, CH_2-α), 4.42 (dd, *J* = 12.0, 3.1 Hz, 1H, one of H-1′), 5.24 (m, 1H, H-2′).

^{13}C NMR (75 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 13.99 (2× C-16), 22.81 (2× C-15), 25.06 (2× C-3), 29.29, 29.51, 29.83, 32.08, 34.22 (C-2), 34.35 (C-2), 54.18 (–$N(CH_3)_3$), 59.56 (C-α), 62.74 (C-1′), 64.07 (C-3′), 66.50 (C-β), 70.52 (C-2′), 173.77 (C-1), 174.14 (C-1).

^{31}P NMR (121 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −0.33.

2.8. General procedure of enzymatic hydrolysis of phosphatidylcholine

Phosphatidylcholine (PC) (0.5 mmol) was suspended in 200 mM Tris–HCl buffer of pH 8.0 (4 ml) containing 80 mM $CaCl_2$. Methylene chloride (8 ml) was added and lastly, 80 U of phospholipase D from *S. chromofuscus*. The reaction mixture was intensively mixed in 35 °C under a nitrogen atmosphere, protected from light. The reaction's progress was monitored by TLC ($CHCl_3$:MeOH:H_2O, 65:25:2, v/v/v) and HPLC. After 16 h, the reaction was quenched with 0.2 M ethylenediamine tetraacetic acid (EDTA) (2.5 ml) and the pH was adjusted to pH < 1 by the addition of 2 M HCl. The reaction mixture was extracted with a mixture $CHCl_3$:MeOH (2:1, v/v, 3× 15 ml). The combined organic phases were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The product was dissolved in MeOH (10 ml) and precipitated from chilled acetone (−15 °C, 50 ml); the solid was filtered off and washed with acetone. For removing calcium ion and to convert phosphatidic acid into its protonated form, the product was dissolved in $CHCl_3$:MeOH:H_2O (5:4:1, v/v/v) and applied to a column of DOWEX 50W X8 (H^+ form) resin. The required product was eluted with the same solvent (100 ml), the solution was evaporated *in vacuo* and the residue was co-evaporated several times from $CHCl_3$:MeOH (2:1, v/v). The solid was lyophilised from benzene to give 1,2-diacyl-*sn*-glycero-3-phosphatidic acid in its protonated form.

2.8.1. 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid (DPPA) (**2a**)

Yield 89%, white solid.

TLC R_f: 0.28 ($CHCl_3$:MeOH:H_2O, 65:25:2, v/v/v), HPLC R_t = 14.5 min.

ESI-MS *m*/*z*: 646.5 $[M-H]^-$.

^{1}H NMR (300 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.88 (t, *J* = 6.7 Hz, 4H, 2× CH_3-16), 1.18–1.36 (m, 48H, 2 × 12 –CH_2–), 1.54–1.71 (m, 4H, 2× CH_2-3), 2.28–2.39 (m, 4H, 2× CH_2-COO), 4.10 (dd, *J* = 6.6, 5.2 Hz, 2H, CH_2-3′), 4.19 (dd, *J* = 12.0, 6.4 Hz, 1H, one of CH_2-1′), 4.38 (dd, *J* = 12.0, 3.6 Hz, 1H, one of CH_2-1′), 5.24 (m, 1H, H-2′).

^{13}C NMR (75 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 14.10 (2× C-16), 22.89 (2× C-15), 25.10 (2× C-3), 29.35, 29.58, 29.90, 32.16, 34.29 (C-2), 34.39 (C-2), 62.57 (C-1′), 64.24 (C-3′), 70.34 (C-2′), 173.79 (C-1), 174.19 (C-1).

^{31}P NMR (121 MHz, $CDCl_3$:MeOD, 2:1, v/v): δ: 0.47.

2.8.2. Phosphatidic acid (PA) from egg-yolk lecithin (**2b**)

Yield 82%, brown oil.

TLC R_f: 0.12 ($CHCl_3$:MeOH:H_2O, 65:25:2, v/v/v), HPLC R_t = 14.3 min.

^{1}H NMR (300 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 5.2 Hz, 8H, 2× CH_3–), 1.24–1.41 (m, 44H, 22× CH_2–), 1.48–1.76 (m, 4H, 2× CH_2-3), 1.92–2.15 (m, 4H, 2× CH_2-CH=CH–), 2.22–2.42 (m, 4H, 2× CH_2-COO), 2.80 (m, 1H, =CH-CH_2-CH=), 4.02–4.13 (m, 2H, CH_2-3′), 4.19 (dd, *J* = 12.0, 6.9 Hz, 1H, one of CH_2-1′), 4.44 (m, 1H, one of CH_2-1′), 5.28 (m, 1H, H-2′), 5.31–5.49 (m, 2H, –CH=CH–).

^{31}P NMR (121 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −1.45.

2.9. Conversion of PA to monopyridynium salt

Conversion of PA to monopyridynium salt was carried out according to the procedure described by Ryu et al. [24]. PA (0.4 mmol) was dissolved in 25 ml of $CHCl_3$:MeOH:pyridine:H_2O (3:3:1:1, v/v/v/v) and applied to a column DOWEX 50W X8 (pyridinium form) resin. The required product was eluted with the same solvent, the solution was evaporated *in vacuo* and the residue was co-evaporated several times from $CHCl_3$:MeOH (2:1, v/v). The solid was lyophilised from benzene to give 1,2-diacyl-*sn*-glycero-3-phosphatidic acid in monopyridinium form (yield > 95%).

2.10. General procedure of studies of coupling reaction of PA with DHEA

Pyridinium salt of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (**4a**) (11 mg, 0.015 mmol) was dissolved in anhydrous pyridine (200 μλ) and to this stirred solution DHEA (8.6 mg, 0.030 mmol) was added followed by immediate addition of coupling agent (0.030 mmol) in dry pyridine (150 μλ). In particular intervals of time, the samples of reaction mixture were taken up and reaction progress was monitored by HPLC.

2.11. General procedure of synthesis of phosphatidyl DHEA analogues

Mixture of pyridinium salt of phosphatidic acid (**4a**) and (**4b**) (0.12 mmol) and DHEA or its 7α-OH analogue (0.08 mmol) was dried by repeated co-evaporation with anhydrous mixture

CH_2Cl_2:benzene (1:1, v/v, 3× 5 ml). The dried residue was dissolved in dry pyridine (1.5 ml) and DCC (49 mg, 0.24 mmol) in 500 ml of dry pyridine was added. After stirring for 48 h, the reaction was quenched with addition of H_2O (100 μl) and then filtered and concentrated *in vacuo*. The oily residue was chromatographed on a silica-gel column with increasing amounts (from 2% to 10%) of CH_3OH in $CHCl_3$. The corresponding fractions (checked on TLC with the primuline test) were collected and evaporated to give the appropriate phosphatide derivative of DHEA. Purity of compounds was determined by HPLC (>98%).

*2.11.1. 3β-O-(1,2-Dipalmitoyl-sn-glycero-3-phospho) -androst-5-en-17-one (DPP-DHEA) (**5a**)*

Yield 74%, white solid.

TLC R_f: 0.29 ($CHCl_3$:MeOH:NH_3 aq., 85:15:2, v/v/v), HPLC R_t = 11.6 min.

ESI-MS *m/z*: $[M-NH_4^+]^-$ 917.7.

^{1}H NMR (600 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 7.0 Hz, 6H, 2× CH_3-16 from acids), 0.91 (s, 3H, CH_3-18), 1.06 (s, 3H, CH_3-19), 1.19–1.44 (m, 48H, 2 × 12× CH_2 from acids), 1.57–1.65 (m, 4H, 2× CH_2-3 from acids), 2.25–2.40 (m, 4H, 2× CH_2-COO from acids), 3.94–4.03 (m, 3H, H-3, CH_2-3′), 4.19 (dd, *J* = 12.0, 6.6 Hz, 1H, one of CH_2-1′), 4.40 (m, 1H, one of CH_2-1′), 5.23 (m, 1H, H-2′), 5.40 (m, 1H, H-6).

^{13}C NMR (150 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 13.76 (C-18), 14.22 (2× C-16 from acids), 19.56 (C-19), 20.65 (C-11), 22.17 (C-15), 22.97 (2× C-15 from acids), 25.22 (2× C-3 from acids), 29.50, 29.67, 30.00, 31.11 (C-7), 31.68 (C-8), 31.82 (C-12), 32.24, 34.43 (C-2 from acid), 34.60 (C-2 from acid), 36.21 (C-16), 36.96 (C-10), 37.41 (C-1), 40.55 (C-4), 48.07 (C-13), 50.57 (C-9), 52.11 (C-14), 62.87 (C-1′), 63.80 (C-3′), 70.82 (C-2′), 76.18 (C-3), 121.79 (C-6), 140.85 (C-5), 173.86 (C-1 from acids), 174.25 (C-1 from acids), 221.2 (C-17).

^{31}P NMR (243 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −2.54.

*2.11.2. 3β-O-(1,2-Diacyl-sn-glycero-3-phospho) -androst-5-en-17-one (P-DHEA) (**5b**)*

Yield 75%, light yellow solid.

TLC R_f: 0.29 ($CHCl_3$:MeOH:NH_3 aq., 85:15:2, v/v/v), HPLC R_t = 11.7 min.

^{1}H NMR (600 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 7.2 Hz, 6H, 2× CH_3-16 from acids), 0.91 (s, 3H, CH_3-18), 1.02 (m, 1H, H-9), 1.05 (s, 3H, CH_3-19), 1.22–1.42 (m, 44H, 22× CH_2 from acids), 1.57–1.65 (m, 4H, 2× CH_2-3 from acids), 1.94–2.17 (m, 4H, 2× CH_2-CH=CH- from acids), 2.27–2.39 (m, 4H, 2× CH_2-COO from acids), 2.78 (m, 1H, =CH-CH_2-CH= from acids), 3.92–4.03 (m, 3H, H-3, CH_2-3′), 4.19 (dd, *J* = 12.0, 6.6 Hz, 1H, one of CH_2-1′), 4.41 (dd, *J* = 12.0, 3.4 Hz, 1H, one of CH_2-1′), 5.23 (m, 1H, H-2′), 5.27–5.37 (m, 2H, –CH=CH– from acids), 5.40 (m, 1H, H-6).

^{31}P NMR (243 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −2.18.

*2.11.3. 3β-O-(1,2-Dipalmitoyl-sn-glycero-3-phospho)-7α -hydroxy-androst-5-en-17-one (DPP-7α-OH-DHEA) (**6a**)*

Yield 57%, white solid.

TLC R_f: 0.23 ($CHCl_3$:MeOH:NH_3 aq., 85:15:2, v/v/v), HPLC R_t = 13.7 min.

ESI-MS *m/z*: $[M-NH_4^+]^-$ 933.7.

^{1}H NMR (600 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 7.0 Hz, 6H, 2× CH_3-16 from acids), 0.90 (s, 3H, CH_3-18), 1.02 (s, 3H, CH_3-19), 1.21–1.35 (m, 48H, 24× CH_2 from acids), 1.55–1.64 (m, 4H, 2× CH_2-3 from acids), 2.28–2.36 (m, 4H, 2× CH_2-COO from acids), 3.92 (m, 1H, H-7), 3.95–4.01 (m, 3H, CH_2-3′, H-3), 4.18 (m, 1H, one of CH_2-1′), 4.40 (dd, *J* = 12.0, 3.4 Hz, 1H, one of CH_2-1′), 5.23 (m, 1H, H-2′), 5.62 (dd, *J* = 5.3, 1.6 Hz, 1H, H-6).

^{13}C NMR (151 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 13.41 (C-18), 14.12 (2× C-16 from acids), 18.35 (C-19), 20.33 (C-11), 22.03 (C-15), 22.90 (2× C-15 from acids), 25.15 (2× C-3 from acids), 29.40, 29.60, 29.92, 30.89, 31.33 (C-2), 32.17, 34.35 (C-2 from acid), 34.50(C-2 from acid), 36.13 (C-16), 37.02 (C-1), 37.62 (C-8, C-10), 40.45 (C-4), 42.60 (C-9), 45.25 (C-14), 47.61 (C-13), 62.82 (C-1′), 63.81 (C-3′), 63.92 (C-7), 70.77 (C-2′), 75.73 (C-3), 124.24 (C-6), 145.32 (C-5), 173.88 (C-1 from acids), 174.25 (C-1 from acids), 221.0 (C-17).

^{31}P NMR (243 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −2.08.

*2.11.4. 3β-O-(1,2-Diacyl-sn-glycero-3-phospho)-7α -hydroxy-androst-5-en-17-one (P-7α-OH-DHEA) (**6b**)*

Yield 59%, light yellow solid.

TLC R_f: 0.23 ($CHCl_3$:MeOH:NH_3 aq., 85:15:2, v/v/v), HPLC R_t = 13.6 min.

^{1}H NMR (600 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: 0.89 (t, *J* = 7.2 Hz, 6H, 2× CH_3– from acids), 0.90 (s, 3H, CH_3-18), 1.03 (s, 3H, CH_3-19), 1.21–1.40 (m, 46H, 22× CH_2 from acids), 1.57–1.65 (m, 4H, 2× CH_2-3 from acids), 2.28–2.36 (m, 4H, 2× CH_2-COO from acids), 2.74–2.90 (m, 1H, =CH-CH_2-CH= from acids), 3.91 (m, 1H, H-7), 3.94 – 4.02 (m, 3H, CH_2-3′, H-3), 4.18 (m, 1H, one of CH_2-1′), 4.42 (m, 1H, one of CH_2-1′), 5.23 (m, 1H, H-2′), 5.29–5.45 (m, 2H, –CH=CH– from acids), 5.62 (dd, *J* = 5.3, 1.5 Hz, 1H, H-6).

^{31}P NMR (243 MHz, $CDCl_3$:MeOD, 2:1, v/v) δ: −1.90.

2.12. Conversion of phosphatides to ammonium salt

Phosphatide was dissolved in $CHCl_3$:MeOH:H_2O (5:4:1, v/v/v) and applied to a column DOWEX 50W X8 (NH_4^+ form) resin. The required product was eluted with the same solvent, the solution was evaporated *in vacuo* and the residue was co-evaporated several times from $CHCl_3$:MeOH (2:1, v/v). The solid was lyophilised from benzene to give a solid residue.

3. Results and discussion

Many promising pharmaceutical agents, displaying high efficacy in *in vitro* studies, are less active or even inactive after *in vivo* application. This situation is most crucial after oral administration [28]. In a similar way, DHEA has many diverse physiological, biological and biochemical effects *in vitro*, but oral DHEA administration produces far more limited clinical effects [4,10,11]. One of the possible solutions to this problem is the conversion of DHEA to active metabolites and/or its transformation into prodrug form.

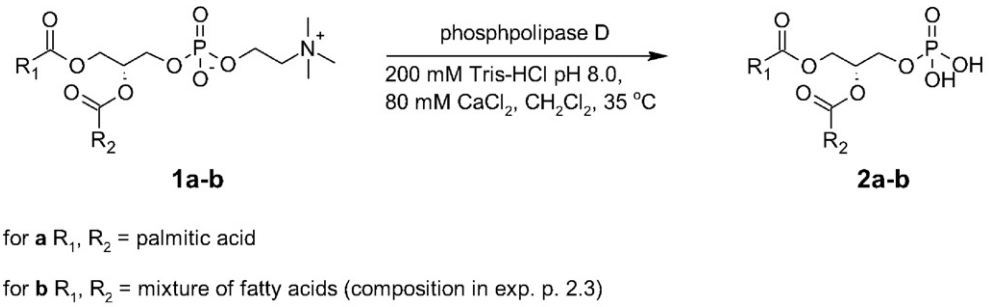
In this article, the synthesis of DHEA analogues modified with phosphatide moiety as a potential prodrugs is described. As a source of phosphatide modification of DHEA and its 7α-hydroxy derivative (7α-OH DHEA), two different phosphatidic acids, DPPA (**2a**) and 1,2-diacyl-*sn*-glycero-3-phosphatidic acid (PA: **2b**), were used. These acids were obtained by the enzymatic hydrolysis of DPPC (**1a**) and PC (**1b**) from egg yolk, respectively (Scheme 1).

The choice of naturally occurring lecithin as a precursor of PA was due to the fact that PC obtained from egg yolk contains polyunsaturated fatty acids (PUFAs), that is, linoleic acid, arachidonic acid and docosahexaenoic acid, which are essential elements of human nutrition and show many biological and medical properties [29–31].

DPPC (**1a**) was obtained according to well-known procedures [32,33] in the reaction of cadmium salt of *sn*-glycero-3-phosphocholine with palmitic acid using as a coupling agent DCC in the presence of 4-(dimethyl-amino)pyridine (DMAP). PC (**1b**) was isolated from egg yolk according to the method described by Palacios and Wang [23].

The enzymatic hydrolysis of PCs (**1a**) and (**1b**) were carried out in a biphasic system by phospholipase D from *S. chromofuscus* with very good yields (89% and 82%, respectively) (Scheme 1).

The key reactions of DHEA and 7α-OH-DHEA with PAs were carried out by the phosphodiester approach, which was often used in the synthesis of phospholipids modifications [15,28] or



Scheme 1. Enzymatic hydrolysis of phosphatidylcholines by PLD *S. chromofuscus*. **1a**: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; **1b**: phosphatidylcholine from egg yolk.

Table 1
Synthesis of DHEA phosphatide (**5a**) with different coupling agents. Composition (according to HPLC) of product mixture after 48 h of the reaction.

Entry	Coupling agent	DPPA pyridinium salt		
		% of **5a**	% of **2a**	% of **7**
1	DCC	68	19	13
2	TsCl	60	35	5
3	MsCl	40	56	4
4	TPSCl	39	56	5

Reaction conditions: DPPA as monopyridinium salt 1 equiv., DHEA 2 equiv., coupling agent 2 equiv., pyridine.

oligonucleotides [34–36] (Scheme 2). We started our experiments according to Williams and Boehm [22] using 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (**2a**) and excess of DHEA in the presence of two equivalents of 2-mesitylene-sulphonyl chloride (MsCl) as a coupling agent in dry pyridine.

Our initial attempts to obtain DHEA modified with PA afforded the desired 3β-*O*-(1,2-dipalmitoyl-*sn*-glycero-3-phospho)-androst-5-en-17-one (**5a**) with a yield of 35%. Because of this low yield, in the subsequent experiments, the pyridinium salt of PA was used as more reactive species in the phosphodiester approach [24,34–36]. Other coupling agents: DCC, *p*-toluenesulphonyl chloride (TsCl) and 2,4,6-tri-isopropylbenzenesulphonyl chloride (TPSCl) were also checked. The reaction progress was monitored by HPLC and the results are presented in Table 1.

The pyridinium salt in comparison with the protonated form of DPPA (**2a**) showed, as expected, higher reactivity. DCC was most effective as a coupling agent in the reaction of PA (**2a**) with DHEA. Comparable yield of product was obtained when TsCl was applied. Poor yield of synthesis of DPP-DHEA (**5a**) was observed in the case of MsCl and TPSCl.

The literature reports that the formation of the phosphate ester with application of DCC as a coupling agent gave rather different results. Oertel and Benes [21] synthesised DPP-DHEA (**5a**) using DCC, but its application by the method of Williams and Boehm [22] was unsuccessful. In our case, DCC was the most efficient coupling agent.

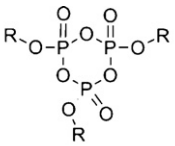


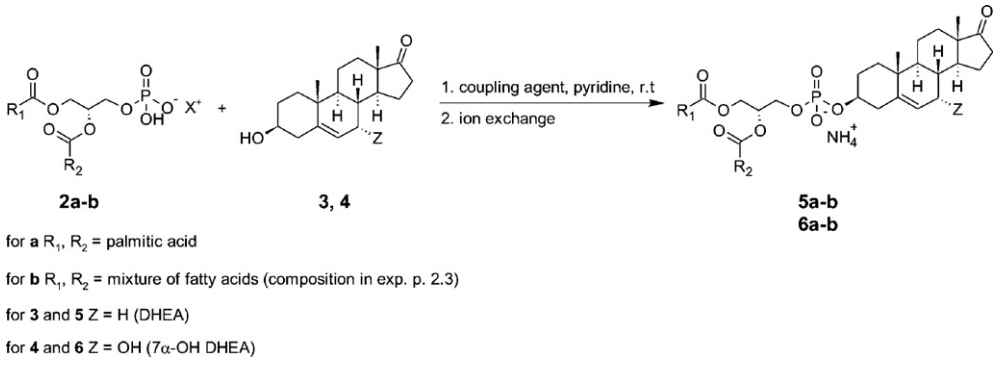
Fig. 1. Structure of trimetaphosphate **7**.

It is noteworthy that in each reaction the presence of unreacted substrate (**2a**) in the product mixture was detected and formation of by-product **7** was observed. On the basis of studies of Khorana and coworkers [35,36] and Todd [34], we assume that product **7** is trimetaphosphate formed as a result of polymerisation of PA (**2a**) (Fig. 1). Formation of a trimetaphosphate **7** in the presence of DCC was confirmed also in the reaction without DHEA. Application of three or four molar equivalents of coupling agent gave comparable yields, eliminated the presence of substrate **2a** but also led to formation of significant amount of by-product **7**. Fortunately, it was easily separated from the product mixture by column chromatography.

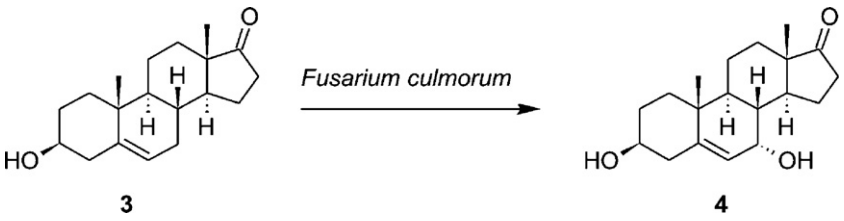
In the synthesis of the phosphatidyl derivatives (**5a**) and (**5b**), the more valuable as a substrate are steroids. On that account, in the next experiments, excess (1.5 equiv.) of PA in relation to DHEA and two molar excess of DCC per acid was used. This procedure led to obtain appropriate phosphatidyl derivatives of DHEA (**5a**) and (**5b**) in good yields (Table 2).

The steroid substrate 7α-OH DHEA (**4**) for the syntheses of appropriate phosphatides (**6a**) and (**6b**) was obtained according to protocol elaborated in our laboratory by microbial biotransformation of DHEA (Scheme 3) [26].

The incorporation of 7α-hydroxy-DHEA (**4**) into corresponding PAs (**2a**) and (**2b**) was achieved with good yields (Table 2). Regios-



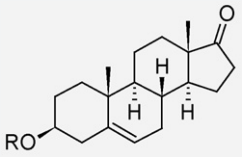
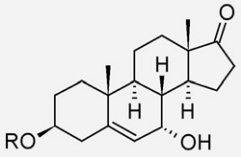
Scheme 2. Synthesis of DHEA and 7α-OH DHEA phosphatides.



3 4

Scheme 3. Synthesis 7α-hydroxy-DHEA **4** by microbial transformation [26].

Table 2
The yields of synthesis of DHEA analogues modified with phosphatidic acids.

Entry	Monopyridinium salt of phosphatidic acid	Isolated yield (%)	
			
1	DPPA	74	57
2	PA from egg yolk	75	59

Reaction conditions: phosphatidic acid as monopyridinium salt 2 equiv., DHEA 1 equiv., coupling agent 3 equiv., pyridine; R: phosphatide.

Table 3
Chemical shifts (δ, ppm) of characteristic atoms of phosphatides of DHEA analogues and their parent compounds.

Compound	CH-6	CH-3		CH-7	CH_3-19	CH_3-18
	δ H	δ H	δ C	δ H	δ H	δ H
Chemical shifts (δ) of sterol part of molecule						
5a	5.40, m	3.94–4.03, m	76.18	–	1.06, s	0.91, s
5b	5.40, m	3.92–4.03, m	–	–	1.05, s	0.91, s
3	5.38 dt, 5.1, 1.6 Hz	3.48, m	71.61	–	1.06, s	0.91, s
6a	5.62 dd, 5.3, 1.6 Hz	3.95–4.01, m	75.73	3.92, m	1.02, s	0.90, s
6b	5.62 dd, 5.3, 1.5 Hz	3.94–4.02, m	–	3.91, m	1.03, s	0.90, s
4	5.65 dd, 5.3, 1.9 Hz	3.59 tdd, 11.2, 4.0, 4.3 Hz	71.21	3.98, m	1.02, s	0.89, s

Compound	CH-2′	One of CH_2-1′	One of CH_2-1′	CH_2-3′	–CH=CH–	CH_2-COO
Chemical shifts (δ) of phosphatide part of molecule						
5a	5.23, m	4.40, m	4.19 dd, 12.0, 6.6 Hz	3.94–4.03, m	–	2.25–2.40, m
5b	5.23, m	4.41 dd, 12.0, 3.4 Hz	4.19 dd, 12.0, 6.6 Hz	3.92–4.03, m	5.27–5.37, m	2.27–2.39, m
6a	5.23, m	4.40 dd, 12.0, 3.4 Hz	4.18, m	3.95–4.01, m	–	2.28–2.36, m
6b	5.23, m	4.42, m	4.18, m	3.94–4.02, m	5.29–5.45, m	2.28–2.36, m
2a	5.24, m	4.38 dd, 12.0, 3.6 Hz	4.19 dd, 12.0, 6.4 Hz	4.10 dd, 6.6, 5.2 Hz	–	2.28–2.39, m
2b	5.28, m	4.44, m	4.19 dd, 12.0, 6.9 Hz	4.02–4.13, m	5.31–5.49, m	2.22–2.42, m

electivity at this esterification at position 3 of 7α-hydroxy-DHEA (**4**) is worth notice. 7-Hydroxy derivative of DHEA (**4**) contains two hydroxyl groups but only one of them reacts with PAs. It is probably an effect of the sterically hindered OH group in the allylic position of 7α-OH DHEA (**4**).

The structure of synthesised derivatives of DHEA and 7α-OH DHEA modified with PAs moiety (**5a**) and (**5b**) and (**6a**) and (**6b**) was confirmed by their spectral data (^{1}H and ^{13}C NMR, mass spectrometry (MS)). Comparison of characteristic signals of starting and modified derivatives of DHEAs are presented in Table 3. In ^{1}H and ^{13}C NMR spectra, signals of both steroid and phosphatide parts of the molecules are visible. The changes were observed in chemical shifts of proton and carbon at position 3 of sterol and their phosphatide. Moreover, in an appropriate modification of DHEA analogues (**5b**) and (**6b**) with PA from egg-yolk lecithin, signals from protons at carbons of double bond (δ = 5.27–5.37 for **5b** and δ = 5.29–5.45 for **6b**) are observed. It confirms the presence of unsaturated fatty acids in the products (**5b**) and (**6b**). It is in agreement with the GC analysis, which indicated that the composition of fatty acids in the products (**5b**) and (**6b**) is that same in substrate (**2b**).

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