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SYNTHESIS AND SPECTRAL AND LUMINESCENCE PROPERTIES OF NEW CONJUGATES OF BRASSINOSTEROIDS FOR IMMUNOFLUORESCENCE ANALYSIS

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Conjugates of 24-epibrassinolide and 24-epicastasterone with indoleacetic acid and nitrobenzofurazan were synthesized. It was shown that the prepared conjugates exhibited effective fluorescence properties and could be used as fluorescent-labeled antigens in immunochemical analysis of brassinosteroids of the 24-epibrassinolide class.

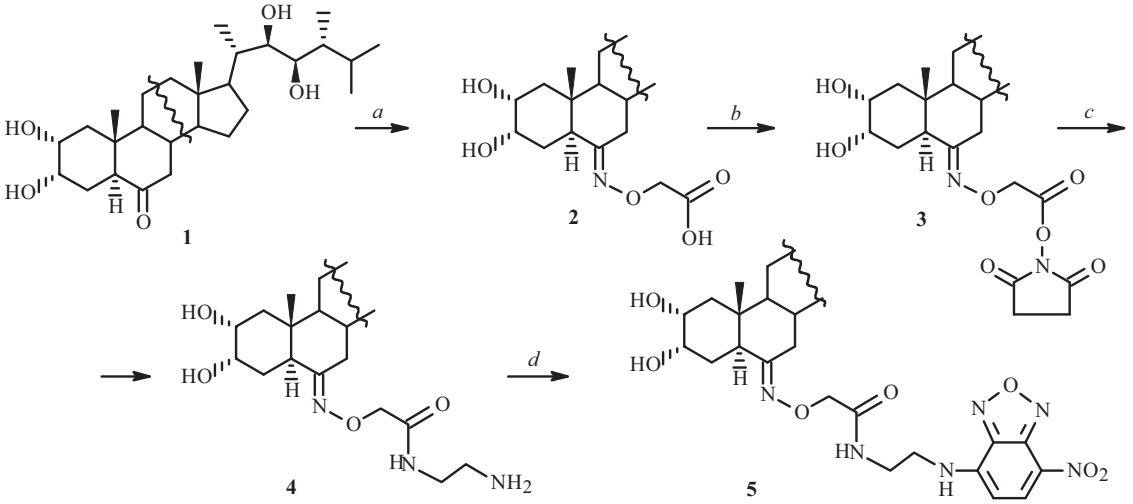
Keywords: brassinosteroids, fluorescent-labeled antigens, synthesis, spectral and luminescence properties.

Practical application of brassinosteroids in agriculture [1] and the prospects of application in medicine necessitate reliable analytical methods for their determination. In this respect, immunoenzyme analytical methods, which do not require multi-step sample preparation, are highly recommended [2]. A common step of these methods is the specific interaction of the determined enzyme-labeled steroid with antibodies. Therefore, the analytical results will be inaccurate under conditions where the steroid conjugate with the enzyme and the determined steroid can be destroyed (e.g., its enzymatic hydrolysis by plant proteinases).

The solution to this problem could be the use of non-proteinaceous compounds as the label. The presence of a fluorescent group in the studied compounds enables them to be detected by instrumental methods based on luminescence measurements. Such a capability was demonstrated earlier using the conjugate of 24-epicastasterone and dansylhydrazine [3].

Herein the synthesis and spectral and luminescence properties of new conjugates of brassinosteroids with various types of luminophores are reported as a continuation of research on the creation of fluorescent probes for immunochemical analysis of this class of compounds [3, 4].

Conjugate **5** of 24-epicastasterone **1** [5] with nitrobenzofurazan was prepared according to Scheme 1.



a. NH_2OCH_2COOH; *b*. NOS/DCC; *c*. $NH_2CH_2CH_2NH_2$; *d*. NBD-Cl/$NaHCO_3$

Scheme 1

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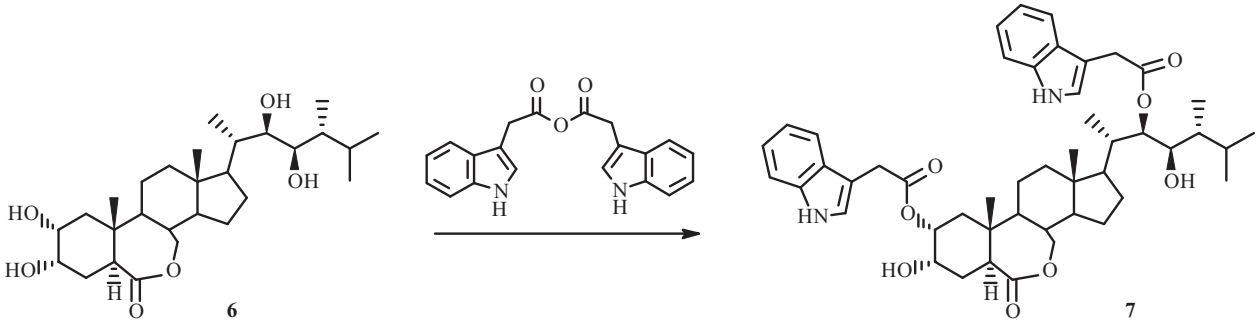
TABLE 1. Spectral and Fluorescence Properties of Brassinosteroids, Fluorescent Labels, and Their Conjugates

Compound	λ^{abs}_{max}, nm	ε, L·mol^{-1}·cm^{-1}	λ^{fl}_{max}, nm	γ	τ, ns
1	286	50	332	10^{-4}	9.9
	240	120			
6	280	20	–	–	–
	240	60			
5	463	21000	535	0.35	6.5
	331	9300			
8	337	10000	–	–	–
7	281	15000	333	0.4	3.5
	221	77000			1.5
9	280	6000	337	0.6	3.3
	221	27000			

λ^{abs}_{max} and λ^{fl}_{max} are the wavelengths of the absorption and fluorescence band maxima of solutions of the studied compounds in EtOH; ε - the extinction coefficient; γ - the quantum yield; τ - the fluorescence lifetime.



In the first step, **1** was converted to 6-(*O*-carboxymethyl)oxime **2** through the reaction with (carboxymethoxy)amine hemi-hydrochloride in Py. Subsequent conversion of **2** into activated *N*-succinimide ester **3** via reaction with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide and reaction of it with ethylenediamine in dioxane gave amine **4**, which gave the desired conjugate **5** upon treatment with an equimolar amount of 7-chloro-4-nitrobenzofurazan.

Conjugate **7** of 24-epibrassinolide **6** with indoleacetic acid was prepared according to the method developed earlier by us for 28-homobrassinosteroids [6] using indoleacetic acid anhydride (three-fold excess) as the acylating agent in dioxane with heating to 40°C in the presence of *N*,*N*-dimethylaminopyridine (DMAP) as a catalyst. The anhydride was prepared by the reaction of indoleacetic acid with dicyclohexylcarbodiimide in anhydrous dioxane.

The spectral and luminescence properties of synthesized conjugates **5** and **7** were compared with those of 7-chloro-4-nitrobenzofurazan (**8**) and indoleacetic acid (**9**).

Figure 1 shows absorption and fluorescence spectra of the new conjugates of 24-epicastasterone (**5**), 24-epibrassinolide (**7**), and their fluorescent labels (**8** and **9**). Table 1 lists the spectral and fluorescence properties of the studied compounds.

The absorption spectrum of the conjugate of 24-epicastasterone **5** (Fig. 1a) differed from that of **8** by the appearance of a broad strong band in the range 400–550 nm with extinction coefficient 21,000 L·mol^{-1}·cm^{-1} at the maximum (λ, 463 nm).

The long-wavelength band in the absorption spectrum of **8**, which was used as the label, was located in the range 300–400 nm with extinction coefficient 10,000 L·mol^{-1}·cm^{-1} at the maximum (λ 337 nm). This band in the spectrum of conjugate **5** practically retained its spectral and energy properties (Table 1). Absorption of 24-epicastasterone did not contribute noticeably to the band of the conjugate because of its low extinction coefficients. 24-Epicastasterone has weak fluorescence and also could not contribute to the fluorescence in the spectral region of the conjugate.

Compound **8** did not fluoresce but became fluorescent upon replacement of the Cl atom [7]. Conjugate **5** fluoresced well. The fluorescence quantum yield was γ = 0.35. The exponential fluorescence decay life-time was τ = 6.5 ns. The fluorescence spectra did not depend on the excitation wavelength. The fluorescence excitation spectra were consistent with the absorption spectra.

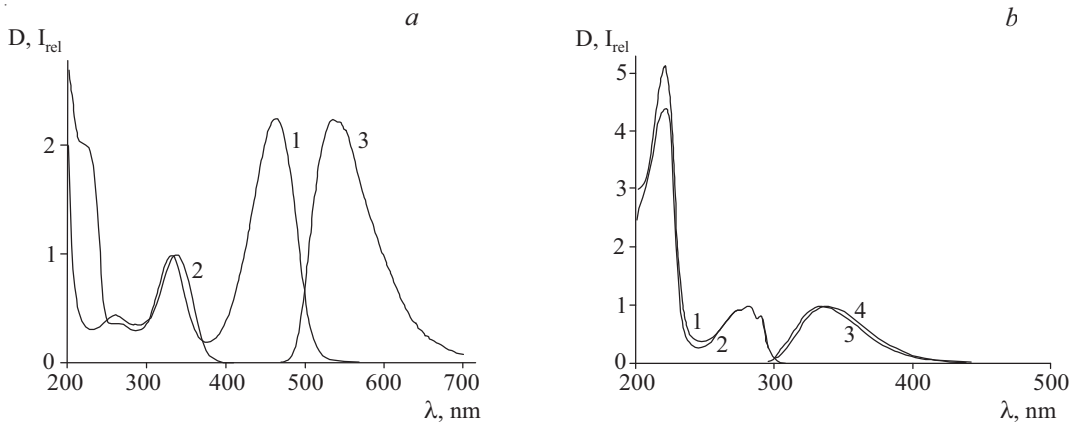


Fig. 1. Absorption spectra of EtOH solutions of conjugates **5** (*a*, 1), **7** (*b*, 1) and compounds **8** (*a*, 2), **9** (*b*, 2); fluorescence spectra of conjugates **5** (*a*, 3), **7** (*b*, 3) and compound **9** (*b*, 4).

The conjugate of 24-epicastasterone with dansylhydrazine that was synthesized by us earlier [3] fluoresced with a similar quantum yield of 0.4. However, its extinction coefficient of 4,600 $L \cdot mol^{-1} \cdot cm^{-1}$ was much less than that of conjugate **5**. This means that performing the fluorescence analysis with new conjugate **5** could increase significantly the sensitivity of the immunochemical determination method for brassinosteroids of the 24-epibrassinolide class.

The absorption spectrum of new 24-epibrassinolide conjugate **7** (Fig. 1b) was practically the same as that of **9**, which was a fluorescence-labeled conjugate. Figure 1b shows absorption spectra normalized to the long-wavelength maxima. The extinction coefficient of 15,000 $L \cdot mol^{-1} \cdot cm^{-1}$ at the long-wavelength maximum (λ 281 nm) of conjugate **7** was more than twice that of **9** (Table 1). The structure of **7** included two molecules of **9**, i.e., the two chromophore molecules were separated by the steroid structure that did not contain conjugated bonds.

Additive absorption of the fragments is usually characteristic of binary chromophores. The fact that the extinction coefficient of conjugate **7** was more than double that of the separate chromophore **9** may be indicative of interchromophore interactions in the conjugate. Absorption of 24-epibrassinolide could not have contributed noticeably to the band of the conjugate because of the low extinction coefficients (Table 1). The fluorescence quantum yield of the conjugate was 0.4. Intramolecular transfer of excitation energy between chromophores was typical of the binary chromophore molecule. Therefore, the emission decay was non-exponential. Extrapolation of the fluorescence decay curve of **7** using two exponents gave emission lifetimes 3.5 and 1.5 ns. Label **9** fluoresced with high quantum yield $\gamma = 0.6$. The emission decayed exponentially with lifetime 3.3 ns.

The fluorescence quantum yield of new conjugate **7** was practically an order of magnitude greater than that of the conjugate of 24-brassinolide with a porphyrin [4]. Solutions of the newly synthesized conjugates had excellent stability and photostability.

The possibility of using the synthesized conjugates as antigen labels for immunofluorimetric determination of brassinosteroids was determined primarily by their immunoreactivity. Binding of the prepared fluorescent-labeled brassinosteroids with antiserum to 24-epicastasterone obtained by us earlier [8] was assessed. The cross reactions of conjugates **5** and **8** with these antibodies were 40 and 5%, respectively. This argued in favor of using **5** as a fluorescent-labeled antigen in immunochemical analysis of brassinosteroids of the 24-epibrassinolide class.

Thus, **5** and **7** exhibited effective fluorescent properties and could act as highly sensitive sensors for studying interactions of phytohormones in biological media.

EXPERIMENTAL

NMR spectra were recorded in $CDCl_3$, CD_3OD, or Py-d_5 solutions on a Bruker Avance DRX-500 instrument (500 MHz for ^1H; 125, ^{13}C). Chemical shifts were determined relative to residual chloroform resonances. Mass spectra were obtained in an LCQ Fleet mass spectrometer. Melting points were measured on a Kofler block. 24-Epicastasterone (**1**) and 24-epibrassinolide (**6**) were synthesized by the literature method [5]. Electronic absorption spectra of solutions of the studied

compounds were recorded on a Cary-500 spectrophotometer. Fluorescence spectra and lifetimes were measured in EtOH at concentration 10^{-5} mol·L^{-1} on Horiba Jobin Yvon and CM-2203 spectrofluorimeters.

(22*R*,23*R*,24*R*)-6-(1-Methoxycarbonyl)imino-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestane (2). A solution of **1** (0.1 g, 0.2 mmol) in Py (3 mL) was treated with a solution of (carboxymethoxy)amine hemi-hydrochloride (0.085 g, 0.67 mmol) in H_2O (1 mL), refluxed for 6 h, poured into H_2O (10 mL), and extracted with $CHCl_3$ (3 × 5 mL). The solvent was evaporated. The solid was chromatographed over silica gel with elution by EtOAc:MeOH (10:1) to afford **2** (0.105 g, 88%), mp 177–178°C (EtOAc:MeOH). IR spectrum (KBr, ν, cm^{-1}): 3460, 2950, 2880, 1720, 1645, 1470, 1450, 1395, 1220, 1110.

PMR spectrum (Py-d_5, δ, ppm, J/Hz): 0.65 (3H, s, 18-Me), 0.90 (3H, s, 19-Me), 0.92 (3H, d, J = 7, 28-Me), 0.96 (3H, d, J = 7, 27-Me), 1.01 (3H, d, J = 7, 26-Me), 1.26 (3H, d, J = 7, 21-Me), 3.65 (1H, m, H-22), 3.99 (1H, dd, J_1 = 4.5, J_2 = 4.5, H-2), 4.06 (1H, m, H-23), 4.40 (1H, m, H-3), 5.01 (2H, s, -O-CH_2-CO).

(22*R*,23*R*,24*R*)-6-[(*N*-Hydroxysuccinimidyl)hydroxymethyl]oximino-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestane (3). A solution of dicyclohexylcarbodiimide (0.029 g, 0.014 mmol) in anhydrous dioxane (0.5 mL) was treated with a solution of carboxymethyloxime (**2**, 0.1 g, 0.19 mmol) and *N*-hydroxysuccinimide (0.019 g, 0.165 mmol) in anhydrous dioxane (2 mL), cooled to 5°C, and stirred for 30 min at 5°C and 20 h at room temperature. The precipitate of dicyclohexylurea was filtered off. The filtrate was evaporated. The solid was dissolved in EtOAc, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to afford **3** (0.1 g, 92%) as an oil. IR spectrum (film, ν, cm^{-1}): 3440, 2950, 2880, 1755, 1730, 1725, 1390, 1215, 1090.

PMR spectrum ($CDCl_3$, δ, ppm): 0.66 (3H, s, 18-Me), 0.74 (3H, s, 19-Me), 0.8–1.00 (12H, m, 21, 26, 27, 28-Me), 2.88 (4H, s, -CO-CH_2-CH_2-CO-), 3.06–3.24 (2H, m), 3.28–3.38 (2H, m), 3.78 (1H, m), 3.92–4.00 (1H, m), 4.88 (2H, m, -O-CH_2-CO).

(22*R*,23*R*,24*R*)-6-[*N*-(2-Aminoethyl)-1-methyloxyacetamide)imino-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestane (4). A solution of **3** (137 mg, 0.2 mmol) in anhydrous dioxane (3 mL) was treated with ethylenediamine (0.3 mL, 4.4 mmol), stirred at room temperature for 2 h, and evaporated. The solid was separated over a column of SiO_2 with elution by EtOAc:MeOH (10:3) to afford **4** (90 mg, 78%) as an oil.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.69 (3H, s, 18-Me), 0.75 (3H, 19-Me), 0.82 (6H, m, 26, 27-Me), 0.89 (3H, d, J = 7, 21-Me), 0.95 (3H, d, J = 7, 28-Me), 2.39 (1H, m, H-5), 2.71 (2H, m, CH_2NH_2), 3.22 (1H, m, H$_\beta$-7), 3.30 (3H, m, H-22, $NHCH_2$), 3.63 (2H, m, H-2, H-23), 3.9 (1H, br.s, H-3), 4.4 (2H, m, O-CH_2-CO).

^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 11.1 q, 12.1 q, 12.7 q, 13.2 q, 17.5 q, 21.4 t, 22.4 q, 24.3 t, 27.1 d, 28.1 t, 30.7 t, 35.8 d, 39.8 t, 40.0 t, 40.5 d, 40.6 s, 41.5 t, 41.6 t, 41.7 d, 43.0 s, 43.6 d, 50.9 d, 52.8 d, 54.3 d, 56.7 d, 68.4 d, 68.7 d, 72.6 t, 72.7 d, 76.4 d, 162.7 s, 171.4 s. Mass spectrum (*m*/*z*) 581 $[M + H]^+$.

Conjugate of 24-Epicastasterone with Nitrobenzofurazan (5). A solution of **4** (80 mg, 0.14 mmol) in MeOH (3 mL) was treated with a saturated solution of $NaHCO_3$ (0.1 mL) and 7-chloro-4-nitrobenzofurazan (30 mg, 0.15 mmol), stirred for 1 d, and evaporated. The solid was separated over a column of silica gel with elution by EtOAc:MeOH (30:1) to afford **5** (54 mg, 52%), mp 123–125°C (MeOH).

PMR spectrum (CD_3OD, δ, ppm, J/Hz): 0.61 (3H, s, 18-Me), 0.65 (3H, 19-Me), 0.83 (6H, m, 26, 27-Me), 0.89 (3H, d, J = 7, 21-Me), 0.93 (3H, d, J = 7, 28-Me), 2.32 (1H, m, H-5), 3.20 (1H, dd, J_1 = 4, J_2 = 13, H$_\beta$-7), 3.30 (1H, m, H-22), 3.33 (2H, s, $NHCH_2CH_2NH$), 3.50 (1H, m, H-2), 3.62 (3H, m, H-23, $NHCH_2CH_2NH$), 3.83 (1H, br.s, H-3), 4.4 (2H, m, O-CH_2-CO), 6.37 (1H, d, J = 9, NBD), 8.45 (1H, d, J = 9, NBD).

^{13}C NMR spectrum (CD_3OD, δ, ppm): 9.9 q, 11.0 q, 11.8 q, 12.0 q, 16.4 q, 20.9 t, 21.4 q, 23.7 t, 26.8 d, 27.6 t, 27.8 t, 30.2 t, 35.5 d, 39.4 t, 39.7 t, 40.0 s, 40.4 d, 41.5 d, 42.6 s, 43.3 d, 52.9 d, 54.2 d, 56.5 d, 68.0 d, 68.4 d, 71.9 t, 72.1 d, 76.0 d, 128.7 d, 130.6 d, 130.7 s, 131.5 s, 143.1 s, 149.7 s, 162.4 s, 172.9 s.

Mass spectrum (*m*/*z*): 743 $[M + H]^+$, 725 $[M + H - H_2O]^+$, 707 $[M + H - 2H_2O]^+$, 579 $[M - NBD + H]^+$, 464 $[M - (OCH_2CONHCH_2CH_2NH\text{-}NBD) + H]^+$.

(22*R*,23*R*,24*R*)-3α,23-Dihydroxy-2α,22-di-(3′-indolylacetoxy)-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (7). A solution of indoleacetic acid (100 mg, 0.62 mmol) in anhydrous dioxane (2 mL) was treated with dicyclohexylcarbodiimide (125 mg) and stirred for 30 min at room temperature. The resulting precipitate of dicyclohexylurea was filtered off. The filtrate was treated with a solution of epibrassinolide (**6**, 50 mg, 0.11 mmol) and DMAP (5 mg) in anhydrous dioxane (2 mL) and stirred for 5 h at 40°C. The solvent was evaporated. The solid was separated over a column of SiO_2 with elution by petroleum ether:EtOAc (1:1) to afford **7** (61 mg, 73%), mp 120–123°C (EtOAc). IR spectrum (film, ν, cm^{-1}): 3400, 1710, 1735, 1620, 760.

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 0.64 (3H, s, 18-Me), 0.76 (3H, s, 19-Me), 0.85–0.90 (6H, m, 21, 26-Me), 0.97 (3H, d, J = 7, 27-Me), 1.01 (3H, d, J = 7, 29-Me), 3.69 (1H, m, H-23), 3.80 (4H, m, CH_2), 4.00 (1H, s, H-3), 4.85 (1H, m, H-2), 5.13 (1H, m, H-22), 7.13–7.21 (8H, m), 7.38 (2H), 7.62 (2H, d, J = 8), 8.51 (2H, s, NH).

^{13}C NMR spectrum ($CDCl_3$, δ, ppm): 11.0 s, 11.6 s, 12.6 s, 15.4 s, 17.5 s, 22.1 d, 22.3 s, 24.8 d, 27.2 t, 27.9 d, 29.0 d, 31.9 d, 37.9 q, 38.6 d, 38.9 t, 39.5 d, 40.4 t, 41.5 s, 41.7 t, 42.5 q, 50.9 t, 52.9 t, 57.0 t, 68.6 t, 69.7 t, 70.3 d, 72.7 t, 76.5 t, 108.4 q, 111.4 t, 111.5 t, 119.1 t, 119.2 t, 119.8 t, 120.0 t, 122.3 t, 122.5 t, 123.3 q, 123.4 q, 127.5 q, 136.3 q, 171.5 q, 175.1 q.

Mass spectrum (*m*/*z*): 829 $[M + H + MeCN]^+$, 795 $[M + H]^+$, 777 $[M + H - H_2O]^+$, 620 $[M + H - IAA]^+$, 445 $[M + H - 2IAA]^+$.

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