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Steroid conjugates: Synthesis and preliminary biological testing of pro-juvenoids

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ABSTRACT

A series of 10 new pro-juvenoids (juvenogens, insect hormonogenic compounds, pro-drug-like agents) was synthesized using isomeric synthetic juvenoids (insect juvenile hormone analogs) and steroid molecules as patterns modifying parts of the complex hormonogenic molecules. In addition, several new synthons were prepared, which were required by the designed synthetic protocol to achieve the target molecules. These pro-juvenoids were subjected to the topical screening tests and to the drinking assays on the red firebug (*Pyrrhocoris apterus*), a convenient model laboratory phytophagous insect. Simple and efficient synthetic procedures for the preparation of the target pro-juvenoids and their synthons are presented. Furthermore, the biological activity of the pro-juvenoids in comparison with the activity of their parent juvenoids and that of several commercially available agents is demonstrated. Juvenoids and pro-juvenoids may replace toxic insecticides persistent in the insect pest control because they have no adverse effects on non-target organisms and/or human.

1. Introduction

The most widespread animals on the Earth, insects (taxonomically order Insecta), also represent one of the most important factors in the ecosystem. A number of insect species became dependent on plants, either as organisms living in symbiosis with plants or pest organisms damaging plants as herbivores, parasites, or pathogens. Plants have developed an efficient defense mechanism, in which pathogenesis-related proteins, polysaccharides and secondary metabolites are responsible for it by providing chemical barriers against animal predators and microbial pathogens.[1] A large number of plant products have proven their biological activity of different types,[2,3] either in their natural forms or in their conjugated forms (glycosides, glycoproteins, etc.), which enable easier transportation of these natural products in plant organisms.[4]

The existence of the insect juvenile hormone (JH) was proven in insects by early experiments on the bug *Rhodnius prolixus*, however, the first JH was isolated and identified later, and the identification of its homologs followed thereafter during a short time period.[5–7] JH's are biosynthesized in the paired endocrine gland *corpora allata* from acetates and propanoates through the mevalonate and homomevalonate pathway.[5] They are released into the hemolymph, and transported to the target cells.[5] They regulate processes of development and reproduction in the insect body physiology in various ways:[5,8–10] (a) insect development and metamorphosis, (b) larval and imaginal diapause, (c) polymorphism, that is, determination of forms (in aphids) or caste determination in social insects, including termites, (d) reproduction, that is, vitelogenesis, and (e) behavior, that is, migration, sexual and copulation behavior and oviposition.

In summary, at least six natural structures of JH's have been isolated and identified up to now.[11,12] Their mode of action at the molecular level has still not been described in full details, however, the observable outcomes of its biological effect are known as a 'juvenilizing effect'.

Insect development and reproduction is a very complex process controlled by insect neurohormones.[8,13] During the developmental cycle, organic molecules are biosynthesized and metabolized in the insect body. The presence and the absence of these molecules are essential for managing a correct course of the insect development. JH influences the transformation of a series of larval instars. The molting hormone (ecdysone) occurs in critical stages of the insect development, and assists in the larval transformation processes. Both hormones are metabolized in the insect body when their presence is no longer needed, and again biosynthesized in the

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appropriate stage of the insect development. It has been proven that the JH concentration is below a measurable level when the last larval instar transforms into the pupa or into the adult. However, if JH is added exogenously at this specific stage of the insect development, the normal developmental cycle is interrupted, and different developmental intermediates occur, which are unable to develop further, reproduce and/or move normally. These so treated individuals either become an easy prey for predators or succumb to their developmental insufficiencies. All these outcomes are the results of biochemical processes, which are influenced by the presence or absence of JH's.[13]

Some of the insect species have become important food competitors of humans and/or vectors of dangerous diseases together with an increase of the human population. Many insect species have migrated to new climatic areas without their natural predators. Often these species become not only food competitors with humans but also vectors of serious diseases. To ensure food resources and health protection, humans have always tried different ways of controlling insect pest population densities. Due to a fast reproduction process in insects, which consists of many generations per year, insects have become more and more resistant to most of the conventional insecticides, which, moreover, have always displayed medium to high toxicity towards warm-blooded animals, fish and different invertebrates.[6] Biodegradation products of insecticides, sometimes displaying much higher toxicity than their parent insecticides themselves, have entered the environment, including the water resources, and have caused important damages and health risk.[14,15] This is one of the key reasons for future restrictions to be put on application of toxic insecticides, for example, organophosphates. Nevertheless, a much more environmentally friendly way of controlling insect pest population density exists in selected areas, based on using insect juvenile hormone bioanalog, JHA's.[5,8,16–19] Some of them have already been on the market for several years, and used against specific insect pests.[7,20–22]

Insect JH's play an important role in the insect development and reproduction cycle.[23] Their natural and synthetic analogs still represent a challenging way for environmentally safe insect pest treatment.[24] Many years ago, we have developed juvenogens (pro-juvenoids, hormonogenic insect pest control agents based on a pro-drug-like application strategy), and since that time, we have synthesized a number of different types of pro-juvenoids in our team, and tested them against non-related insect pests.[6,16,25–27]

The main advantages of pro-juvenoids consist of: (a) their ability to liberate a biologically active component (juvenoid) under the biotic and/or abiotic factors during a longer period of time in a low concentration, in comparison to an instant application of juvenoid itself,[6,16] (b) their mode of action resulting in a more effective and more targeted treatment of the tested insect species than by the biologically active juvenoid, and (c) representing a type of practical formulation of the biologically active juvenoid, which is designed and synthesized in a way enabling controlled enzymatic degradation of its molecule in the insect body resulting in a slow activation of the biologically active compound during a certain period of time. In contrary, the application of the total quantity of juvenoid topically in one portion results in a rapid enzymatic deactivation of juvenoid in the insect body. Pro-juvenoids are designed both for oral and topical applications to insects, even if they are usually bulky molecules, and their penetration through the insect cuticule may be difficult in topical screening tests. However, the structure of the non-juvenoid part of the complex pro-juvenoid molecule may substantially contribute to the most effective way of treating the target insect pests. Considering all these aspects, pro-juvenoids should be more advantageous insect pest control agents in screening tests than their parent juvenoids.

In turn, important disadvantages of juvenoids and pro-juvenoids should also be mentioned. Due to their mode of action, these compounds do not kill the target insects immediately or within a short time. Their effect is slow in comparison with that of conventional insecticides, and they mostly affect the generation of the target insect following that one, which has been treated by a juvenoid, by reducing its population density in the treated area.[7]

In past, we have synthesized different series of pro-juvenoids (fatty acid esters,[9,26] glycosides,[16,27] glyceride derivatives,[28] and finally conjugates of selected juvenoids with bile acids[25]), and tested them against different insect pests. Some of these compounds proved that a pro-juvenoid does display higher biological activity than its parent juvenoid.

The objective of the present research consisted in (a) the synthesis of conjugates of selected juvenoids with bile acids and phytosterols, (b) screening tests of the prepared pro-juvenoids on the red firebug (*Pyrrhocoris apterus*), and (c) the evaluation of the results, including conclusion for the structure–activity relationship.

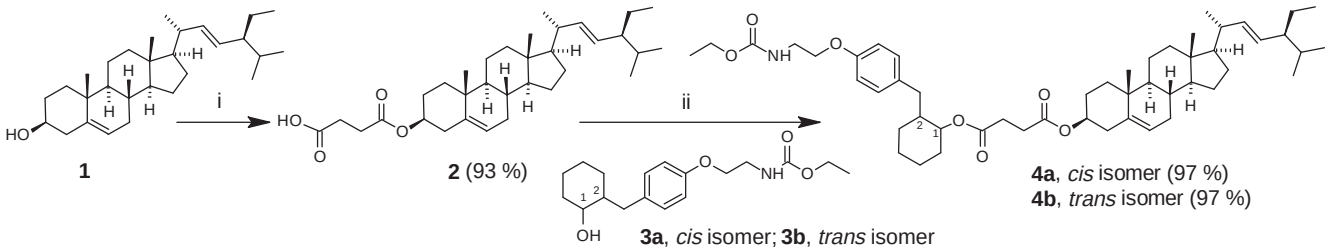
2. Results and discussion

A series of 10 new compounds, which display a mode of action analogous to that of natural insect JH's, has been designed and synthesized. The prepared compounds belong into the category of pro-juvenoids. They always consist of a biologically active insect pest control agent (a juvenoid, an insect juvenile hormone analog) and a component that modifies physico-chemical properties of the target pro-juvenoid and represents an important factor in its practical applicability as an insect pest control agent.

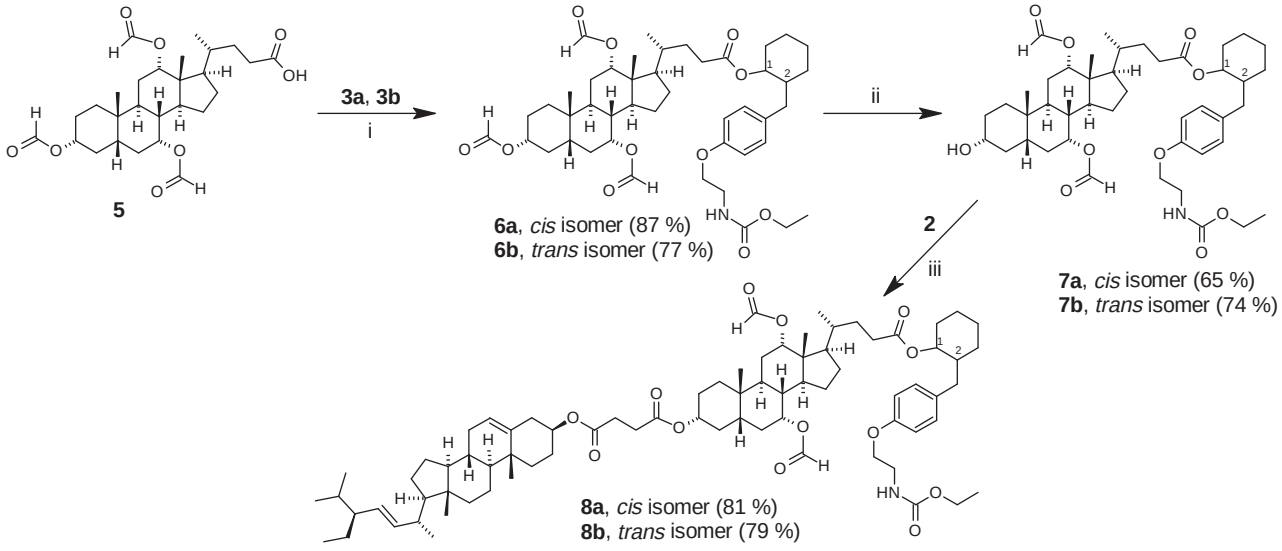
The juvenoids (**3a** and **3b**) used in this investigation were already synthesized earlier,[29] and their synthesis has been modified and improved several times.[30,31] High biological activity of this series of juvenoids brought them to the top of importance among these types of insect pest control agents. To design the presented series of conjugated pro-juvenoids, steroid compounds (derivatives of cholic acid, a typical animal steroid, and stigmasterol, an example of phytosterols) have been selected for their natural origin. During biotic or abiotic degradation of any pro-juvenoid of this series, only synthetic insect pest control agents liberate together with natural products, bile acids or phytosterols.

The first of the synthetic precursors of the target conjugates, 4-oxo-4-[(3β,22*E*)-stigmasta-5,22-dien-3-yloxy]butanoic acid (**2**), was prepared by the reaction of stigmasterol (**1**) with succinic anhydride in pyridine in 93% yield. Isomeric pro-juvenoids **4a** and **4b**, derived from stigmasterol (**1**), were prepared by the reaction of **2** with either of the isomers **3a** or **3b** by means of DCC and under catalysis with 4-pyrrolidinopyrridine in dry benzene in almost quantitative yields (Scheme 1).

The hydroxylic functionalities in cholic acid were protected as formates,[32] the resulting (3α,5β,7α,12α)-3,7,12-tris(formyloxy) cholan-24-oic acid (**5**) was transferred into (3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oyl chloride (by means of oxalyl chloride), and the resulting acyl chloride was directly allowed to react with the *cis* juvenoid alcohol **3a**, affording 2-(4-{2-[(ethoxycarbonyl)amino]ethoxy}benzyl)cyclohexyl (3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oate (**6a**) in 77% yield (Scheme 2). The opposite isomer **6b** could not be prepared by the same synthetic protocol, because no reaction occurred. Therefore, (3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oic acid (**5**) was esterified with **3b** by means of DCC and under the catalysis with DMAP, and **6b** was obtained in 87% yield. In further synthesis, the protecting formyl group at C(3) was removed by using sodium bicarbonate in methanol to give isomeric pro-juvenoids **7a** (74%) or **7b** (65%). In the following synthetic strategy, **7a** and **7b** were conjugated with **2** by DCC under the catalysis of DMAP, affording more complex conjugated pro-juvenoid structures **8a** and **8b** in 80% yields (Scheme 2).

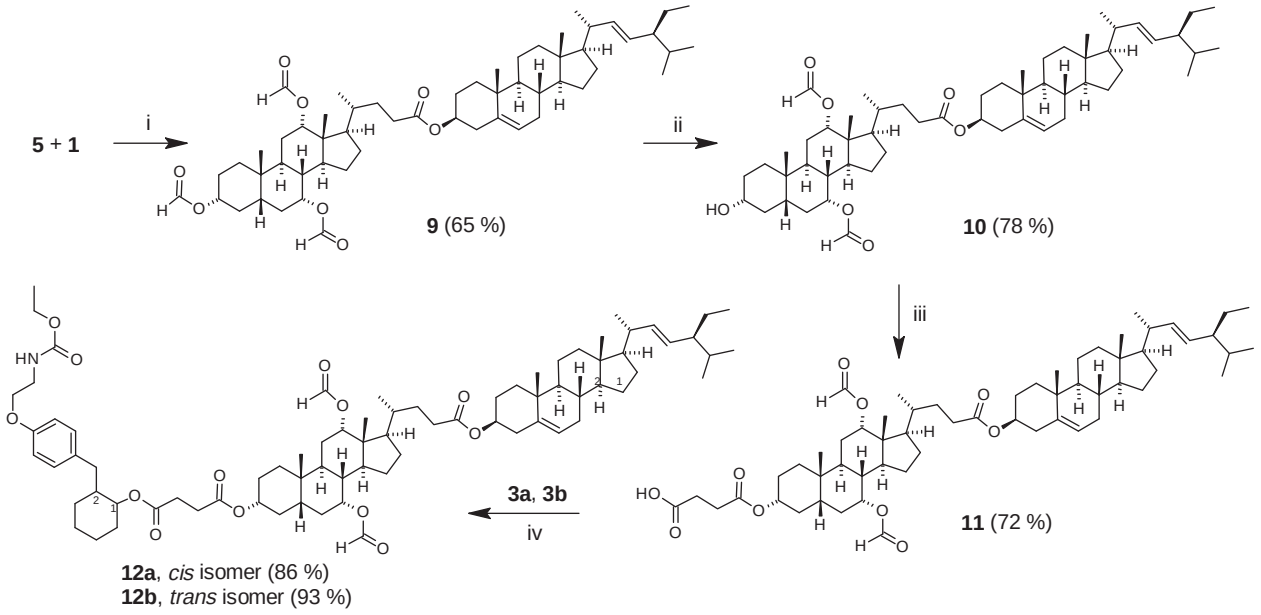


Scheme 1. Synthetic protocol I. Reagents and conditions: (i) succinic anhydride, DMAP, pyridine, 7 days; (ii) DCC, 4-pyrrolidinopyridine, benzene, 48 h.



Scheme 2. Synthetic protocol II. Reagents and conditions: (i) *cis* isomer: (a) oxalyl chloride, benzene, 45 min; (b) **3a**, pyridine, benzene, 24 h; *trans* isomer: **3b**, DCC, DMAP, benzene, 48 h; (ii) $NaHCO_3$, CH_3OH, 19 h; (iii) DCC, 4-pyrrolidinopyridine, benzene, 48 h.

To obtain pro-juvenoids analogous to **8a** and **8b** with the reverse positions of steroid units in the target conjugate molecules (**12a** and **12b**; Scheme 3), the synthetic procedure started with the synthesis of convenient synthon **9**, which was synthesized from stigmasterol (**1**) and (3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oic acid (**5**) in 65% yield. Removing of the protecting



Scheme 3. Synthetic protocol III. Reagents and conditions: (i) DCC, DMAP, benzene, 3 days; (ii) $NaHCO_3$, CH_3OH, 5 h; (iii) succinic anhydride, DMAP, pyridine, 4 days; (iv) DCC, 4-pyrrolidinopyridine, CH_2Cl_2, 48 h.

formyl group at C(3) resulted in achieving **10** in a 80% yield. A short linker was introduced to **10** by means of succinic anhydride, following the earlier mentioned synthetic protocol, affording **11** in a 72% yield. Final conversion of **11** into **12a** (86%) and **12b** (93%) was achieved by the esterification of **11** with either **3a** or **3b** by means of DCC/4-pyrrolidinopyrridine protocol in dry benzene.

Examples of the results of the screening tests of several selected conjugated pro-juvenoids on the red firebug (*P. apterus*) are summarized in Table 1. An application of the conjugates on the red firebug was made according to the standard screening procedures.[8,33] In the topical screening tests, the compounds were dissolved in acetone in three concentrations (0.05, 0.5, and 5 $\mu g\ \mu L^{-1}$), and the resulting solution (1 μL) was applied on the top of freshly molted nymph of the fifth instar of *P. apterus* by using Burkhard microapplicator. Pure acetone was used to treat insects in the reference experiment. In the oral screening tests, application of the pro-juvenoids was made by a drinking assay according to an already published methodology.[34] Each compound was dissolved in acetone (200 μL) and a solution was added into a mixture of a distilled water (50 mL) and Tween-80 (5 μL) to give concentrations of the pro-juvenoid 0.025, 0.25, 2.5, and 25 $\mu g\ \mu L^{-1}$. The resulting solutions were offered at the end of the fourth nymphal instar of *P. apterus*. A mixture of acetone, distilled water, and Tween-80 was used in the reference experiment. In both types of the biological assays, each concentration of the tested compound was applied to 10 individuals and all experiments were performed in three replications. The tested insects were put into Petri dishes and they were kept in a climatic box under artificial lighting (16L:8D) at a temperature 25 ± 0.5 °C and at a relative humidity 50 ± 5%. The development and mortality of the tested insects were checked every day. The evaluation of morphological effects of juvenoids was made individually, according to the degree of metamorphosis inhibition determined by morphological criteria after the next molt. The usual 0% to 100% scoring system was used. Therefore 0% indicates formation of morphologically perfect adults; 20%, 40%, 60%, and 80% indicate intermediates between adult and larval forms (adultoids); and 100% indicates appearance of perfect supernumerary larvae.

For linearization of dose–response curve were average values of morphological effect transformed to probits at each concentration level. The significance of linear regression was tested using analysis of variance and then ID-50 values ($\mu g\ ind^{-1}$) were computed. Differences in JH activities of juvenoids were tested by comparing of their linear regression parameters. One-way ANOVA Fischer's LSD ($P = 0.05$) for determination of differences in drinking assay, residual test and residual test after 80 days was used. The linear regression statistics were computed according to Zar18 and free student version of S-PLUS for Fischer's LSD multiple comparison was used.[33] The resulting biological activity was evaluated according to the degree of inhibition of metamorphosis determined by morphological changes after the last ecdysis.[8,33] The ID-50 value represents a dose of the compound in μg per individual which is responsible for 50% inhibition of metamorphosis. They were calculated by the linear regression after linearization of the dose–response curves using the probit transformation. The results of the screening tests are summarized in Table 1, from which it is evident that the *cis*-isomers (**4a**, **6a**, and **7a**) display much higher biological activity both in topical tests and in drinking assays that the corresponding *trans* isomers (**4b**, **6b**, and **7b**). The finding is in agreement with the results of the screening tests of the parent juvenoids **3a** and **3b**. The stigmasterol-based conjugated pro-juvenoid **4a** was the most active compound from the tested series of conjugates in topical screening tests, but it was very low active in drinking assays. This is understandable if the polarity of the compound is considered (calculated solubility of **4a** in water is only 5.1683×10^{-9} mg mL^{-1}). It can be demonstrated with **6a** that this pro-juvenoid displayed higher biological activity for the drinking assay than for the topical screening test. It is not always easy to demonstrate that a capability of the enzymic system of the tested insect is able to do subsequent metabolizing of the conjugate molecule to liberate the biologically active juvenoid during certain time space. Based on that finding, one can conclude that the tested insects were subjected to longer exposition time with the biologically active compound and, therefore, the biological activity of **6a** found for the drinking assay is higher in comparison with the topical testing of the same pro-juvenoid conjugate.

Solubility of any studied pro-juvenoid seems not to be a key factor for explaining low or no activity of the compound found during the investigation (cf. Table 1). The calculated solubility (based on the partition coefficient, log *P*) of the pro-juvenoids **4a**/**4b**, **6a**/**6b**, and **7a**/**7b** in water is 5.1683×10^{-9} mg mL^{-1}, 5.4789×10^{-8} mg mL^{-1}, and 7.0079×10^{-7} mg mL^{-1}, respectively, and according to the other calculated value, distribution coefficient (log *D*), it is stable within a wide range of pH, which covers the range of pH values which may be reached in the insect gastrointestinal tract. Comparing the biological activity values found for the compounds **4a**/**4b** and **7a**/**7b** clearly demonstrate that the biological activity is not a function of compound solubility in water.

The results presented in Table 1 also demonstrate the advantage of several pro-juvenoid compounds over the commercially available juvenoids, methoprene, pyriproxyfen, and fenoxycarb, which were taken as reference compounds. In topical tests, the pro-juvenoids **4a**, **6a**, and **7a** displayed biological activity comparable with methoprene and fenoxycarb. In drinking assays, the pro-juvenoid **6a** was even more active than methoprene or fenoxycarb.

The data summarized in Table 1 proved again that at least some of the tested pro-juvenoids are able to display a higher biological activity in the comparison with their parent juvenoids. However, it is obvious that the bacterial flora in the insect gut is essential—among others—for food digestion, nutrition, pheromone production, regulation of pH, synthesis of vitamins, resistance against bacterial entomopathogens, detoxification of unnatural compounds, etc. Enzymes produced by the insect intestinal microbiota are important for detoxification of insecticides. We have made a search for a spectrum of cultivable bacterial species in the insect intestinal tract of *P. apterus*, and found that a majority of the isolated bacteria are common for gastrointestinal tract. However, several host-specific bacterial species have also been found.[35]

Another question to be answered is that one, if sterol molecules present in pro-juvenoid structures may be co-responsible for the biological effect of the tested pro-juvenoids. Their effect cannot be excluded completely, because it is known that phytosterols can be transformed into cholesterol, that is, the biosynthetic

Table 1
Results of topical screening and drinking assay of selected juvenoids and pro-juvenoids on *Pyrrhocoris apterus*

Compound	Topical screening		Drinking assay	
	ID-50[a]	r^2[b]	ID-50[a]	r^2[b]
3a	0.001	0.94	0.0012	0.99
3b	0.71	0.93	0.5	0.94
4a	0.0185	0.84	4.989	0.93
4b	>5	SNE[c]	>25	SNE[c]
6a	0.094	0.93	0.051	0.94
6b	1.775	0.91	6.153	0.93
7a	0.062	0.92	0.423	0.99
7b	>5	SNE[c]	>25	SNE[c]
Methoprene	0.01	0.99	0.41	0.98
Pyriproxyfen	2.3	0.91	25	0.92
Fenoxycarb	0.09	0.98	0.2	0.96

[a] Efficacy given in ID-50 values (μg per individual).
[b] r—regression reliability.
[c] SNE—statistically not evaluated.

precursor of ecdysone, insect molting hormone.[36,37] During the evaluation of the effect of the tested compounds, no abnormalities were observed, and, therefore, the effects of all compounds tested were evaluated as the effect identical with the effect of juvenile hormone. Moreover, it has also been published that juvenoids do not affect directly the activity of the ecdysteroid receptor complex.[38] In turn, we had also studied biodegradation of selected juvenoids in the insect body using radiolabeled compounds,[39,40] and came to a final results that juvenoids are metabolized into small molecules with no biological activity related to that one described as 'juvenilizing effect'.

To conclude, the synthesis of the target pro-juvenoid conjugates was achieved by simple synthetic steps, which are valuable especially for possible practical application of the target conjugates for treating insects under the field conditions. The biological activity of the *cis* and *trans* isomers (**4a**, **6a**, and **7a** vs **4b**, **6b**, and **7b**) differ quite substantially. This finding may have a basis either in different accessibility of the respective isomers to the receptors responsible for activation of the biologically active juvenoids or in different ability of the insect enzymes to metabolize the *cis*- and *trans*-derived juvenoid alcohols.

3. Experimental part

3.1. General

The ^{1}H NMR and the ^{13}C NMR spectra were recorded on a Bruker AVANCE 600 MHz spectrometer at 600.13 MHz and 150.90 MHz in deuteriochloroform using tetramethylsilane ($\delta = 0.0$) as internal reference. ^{1}H NMR data are tabulated in the following order: chemical shift (δ) expressed in ppm, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in Hertz, number of protons. Infrared spectra were measured with a Nicolet 205 FT-IR spectrometer. Mass spectra were measured with a Waters ZMD mass spectrometer in a positive ESI mode. TLC was carried out on silica gel plates (Merck 60F254) and the visualization was performed by spraying with the methanolic solution of phosphomolybdic acid (5%) followed by heating. Elemental analyses were performed on a Perkin Elmer 2400, series II CHNS/O analyzer (USA). Melting points were determined on a Kofler MHK melting point apparatus (Franz Küstner Nacht, KG, Dresden, Germany) and are uncorrected. All chemicals and solvents were purchased from regular commercial sources in analytical grade and the solvents were purified by general methods before use. For column chromatography, silica gel 60 (0.063–0.200 mm) from Merck was used. An ACD/Labs software, version 12.01, was used for calculation of solubility, partition coefficient (log *P*) and distribution coefficient (log *D*) of the prepared compounds in water.

3.2. Preparation and characterization of the substances

To have a simple system for assigning signals in the ^{1}H and ^{13}C NMR spectra, the following system has been used in this paper: carbon atoms numbers belonging to the cholic acid molecules and its substructures are not primed, those belonging to the (3β,22*E*)-stigmasta-5,22-dien-3-ol (stigmasterol) molecule and its substructures are single primed and those belonging to the juvenoid molecule and its substructures are double primed. In the case the compound was obtained in non-crystalline form, no melting point is given.

3.3. 4-Oxo-4-[(3β,22*E*)-stigmasta-5,22-dien-3-yloxy]butanoic acid (2)

4-Dimethylaminopyridine (0.20 g, 1.637 mmol) was added to a solution of stigmasterol (**1**; 4.00 g, 9.693 mmol) and succinic anhydride (1.52 g, 15.188 mmol) in dry pyridine (17.5 mL). The mixture was stirred at room temperature for 7 days, and then poured onto a mixture of ice (30 g) and a 37% HCl solution. The aqueous phase was extracted with chloroform (5 × 40 mL), the extract was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with petroleum ether/diethyl ether (3:1) as an eluent to give the compound **2** as a white solid (4.62 g, 9.010 mmol, 93% yield): mp 151–153 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-18′), 0.79 (d, 3H, J = 6.6 Hz, H-27′), 0.80 (t, 3H, J = 7.4 Hz, H-29′), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.95 (ddd, 1H, J = 5.1, 10.9, 12.1 Hz, H-9′), 1.01–1.06 (m) + 1.50–1.56 (m, 2H, H-15′), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 1.06–1.11 (m) + 1.96–2.00 (m, 2H, H-12′), 1.07 (ddd, 1H, J = 6.2, 11.3, 12.6 Hz, H-14′), 1.09–1.12 (m) + 1.83–1.88 (m, 2H, H-1′), 1.12–1.17 (m, 1H, H-17′), 1.14–1.19 (m) + 1.41–1.46 (m, 2H, H-28′), 1.21–1.28 (m) + 1.70 (ddd, 2H, J = 6.1, 9.8, 13.0 Hz, H-16′), 1.37–1.41 (m) + 1.93–2.01 (m, 2H, H-7′), 1.46–1.51 (m, 2H, H-11′), 1.46–1.51 (m, 1H, H-8′), 1.51–1.55 (m, 1H, H-25′), 1.52–1.56 (m, 1H, H-24′), 1.55–1.60 (m) + 1.80–1.86 (m, 2H, H-2′), 2.00–2.06 (m, 1H, H-20′), 2.30–2.33 (m, 2H, H-4′), 2.61 (m, 2H, H-31′), 2.68 (m, 2H, H-32′), 4.63 (dddd, 1H, J = 4.2, 7.1, 9.5, 11.6 Hz, H-3′), 5.01 (dd, 1H, J = 8.9, 15.2 Hz, H-23′), 5.15 (dd, 1H, J = 8.7, 15.2 Hz, H-22′), 5.37 (ddt, 1H, J = 1.3, 1.3, 1.8, 5.0 Hz, H-6′). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.03 (q, C-18′), 12.25 (q, C-29′), 18.97 (q, C-27′), 19.30 (q, C-19′), 20.99 (t, C-11′), 21.09 (q, C-26′), 21.22 (q, C-21′), 24.34 (t, C-15′), 25.40 (t, C-28′), 27.66 (t, C-2′), 28.89 (t, C-16′), 28.91 (t, C-32′), 29.20 (t, C-31′), 31.81 (t, C-7′), 31.87 (t, C-8′), 31.87 (d, C-25′), 36.92 (t, C-1′), 36.56 (s, C-10′), 37.98 (t, C-4′), 39.59 (t, C-12′), 40.51 (d, C-20′), 42.17 (s, C-13′), 49.98 (d, C-9′), 51.21 (d, C-24′), 55.88 (d, C-17′), 56.75 (d, C-14′), 74.53 (d, C-3′), 122.72 (d, C-6′), 129.24 (d, C-23′), 138.31 (d, C-22′), 139.49 (s, C 5′), 171.53 (s, C-30′), 177.54 (s, C-33′). IR (KBr): 1732, 1715, 1381, 1178 cm^{-1}. Anal. Calcd $C_{33}H_{52}O_4$: C, 77.29; H, 10.22. Found: C, 77.15; H, 10.41. MS (ESI, 20 eV): $[M+Na]^+$ 535.

3.4. (*cis*)- and (*trans*)-2-(4-{2-[(ethoxycarbonyl)amino]ethoxy}benzyl)cyclohexyl (3β,22*E*)-stigmasta-5,22-dien-3-yl butanedioate (4a and 4b)

N,*N*′-Dicyclohexylcarbodiimide (0.189 mmol) and 4-pyrrolidinopyridine (0.044 mmol) were added to a mixture of **2** (80 mg, 0.156 mmol) and either **3a** or **3b** (0.202 mmol; prepared according to[29–31]) in dry benzene (4 mL). The resulting mixture was stirred for 2 days at room temperature, and then the solvent was removed under reduced pressure. The crude product was washed by diethyl ether and water. The aqueous phase was extracted by diethyl ether. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The product was purified on a silica gel column with petroleum ether/diethyl ether (3:1 → 1:1) as an eluent to give the compound **4a** (97% yield) or **4b** (97% yield). **4a**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.70 (s, 3H, H-18′), 0.80 (d, 3H, J = 6.5 Hz, H-27′), 0.81 (t, 3H, J = 7.4 Hz, H-29′), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.5 Hz, H-21′), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.39 (dd, 1H, J = 7.9, 13.7 Hz, H-7″), 2.55 (dd, 1H, J = 7.0, 13.7 Hz, H-7″), 3.57 (br q, 2H, J = 5.5 Hz, H-13″), 4.00 (t, 2H, J = 5.1 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.61–4.68 (m, 1H, H-3′), 4.92 (dt, 1H, J = 2.2, 2.2, 4.3 Hz, H-2″), 5.02 (dd, 1H, J = 8.9, 15.1 Hz, H-23′), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.36 (m, 1H, H-6′), 6.81 (m, 2H, H-10″), 7.01 (m, 2H, H-9″). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.25 (q, C-29′), 14.61 (q, C-16″), 18.96 (q, C-27′), 19.29 (q, C-19′), 20.79 (t, C-4″), 20.98 (t, C-11′), 21.09 (q, C-21′), 21.20 (q, C-21′), 24.33 (t, C-15′), 25.02 (t, C-5″), 25.40 (t, C-28′), 26.96 (t, C-6″), 27.73 (t, C-2′), 28.91 (t, C-16′), 29.60 (t, C-31′), 29.60 (t, C-32′), 29.89 (t, C-3″), 31.81 (t, C-7′), 31.86 (d,

C-8′), 31.86 (d, C-25′), 36.57 (s, C-10′), 36.91 (t, C-1′), 37.70 (t, C-7″), 38.07 (t, C-4′), 39.58 (t, C-12′), 40.50 (d, C-20′), 42.17 (s, C-13′), 43.55 (d, C-1″), 50.01 (d, C-9′), 51.21 (d, C-24′), 55.87 (d, C-17′), 56.74 (d, C-14′), 56.77 (d, C-14′), 60.90 (t, C-15″), 72.37 (d, C-2″), 74.33 (d, C-3′), 114.21 (d, C-10″), 122.68 (d, C-6′), 129.23 (d, C-23′), 130.02 (d, C-9″), 132.94 (s, C-8″), 138.30 (d, C-22′), 139.54 (s, C-5′), 139.56 (s, C-5′), 156.69 (s, C-14″), 171.65 (s, C-33′), 171.72 (s, C-30′). IR (KBr): 3362, 1732, 1704, 1666, 1512, 1381, 1242, 1163 cm^{-1}. MS (ESI, 40 eV): $[M+Na]^+$ 839. **4b**: ^1H NMR (600 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-18), 0.79 (d, 3H, J = 6.6 Hz, H-27), 0.81 (t, 3H, J = 7.4 Hz, H-29), 0.85 (d, 3H, J = 6.5 Hz, H-26), 1.00 (s, 3H, H-19), 1.25 (t, 3H, J = 7.1 Hz, H-16′), 2.20 (dd, 1H, J = 9.1, 13.6 Hz, H-7′), 2.83 (dd, 1H, J = 3.9, 13.6 Hz, H-7′), 3.57 (br q, 2H, J = 5.4 Hz, H-13′), 4.01 (t, 2H, J = 5.1 Hz, H-12′), 4.12 (q, 2H, J = 7.1 Hz, H-15′), 4.55–4.65 (m, 1H, H-3), 4.55–4.65 (m, 1H, H-1′), 5.02 (dd, 1H, J = 8.8, 15.1 Hz, H-23), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22), 5.34 (dq, 1H, J = 1.5, 1.5, 1.5, 5.0 Hz, H-6), 5.36 (dq, 1H, J = 1.5, 1.5, 1.5, 5.0 Hz, H-6), 6.80 (m, 2H, H-10′), 7.03 (m, 2H, H-9′). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.25 (q, C-29′), 14.62 (q, C-16″), 18.95 (q, C-27′), 19.27 (q, C-19′), 20.97 (t, C-11′), 21.09 (q, C-26′), 21.20 (q, C-21′), 24.33 (t, C-15′), 24.45 (t, C-4″), 25.40 (t, C-28′), 25.00 (t, C-5″), 27.67 (t, C-2′), 27.71 (t, C-2′), 28.90 (t, C-16′), 29.50 (t, C-31′), 29.54 (t, C-32′), 29.67 (t, C-6″), 30.66 (t, C-3″), 30.72 (t, C-3″), 31.80 (t, C-7′), 31.86 (d, C-8′), 31.86 (d, C-25′), 36.55 (s, C-10′), 36.58 (s, C-10′), 36.91 (t, C-1′), 37.83 (t, C-7″), 38.03 (t, C-4′), 39.58 (t, C-12′), 40.50 (t, C-13″), 40.53 (d, C-20′), 42.16 (s, C-13′), 43.77 (d, C-1″), 49.97 (d, C-9′), 50.01 (d, C-9′), 51.21 (d, C-24′), 55.87 (d, C-17′), 55.89 (d, C-17′), 56.74 (d, C-14′), 56.77 (d, C-14′), 60.91 (t, C-15″), 66.92 (t, C-12″), 74.28 (d, C-3′), 74.60 (d, C-3′), 77.13 (d, C-2″), 114.10 (d, C-10″), 122.65 (d, C-6′), 129.23 (d, C-23′), 130.18 (d, C-9″), 130.29 (d, C-9″), 132.75 (s, C-8″), 138.30 (d, C-22′), 139.53 (s, C-5′), 139.56 (s, C-5′), 154.00 (s, C-11″), 156.65 (s, C-14″), 171.70 (s, C-33′), 172.00 (s, C-30′). IR (KBr): 3354, 1732, 1705, 1665, 1512, 1381, 1241, 1165 cm^{-1}. MS (ESI, 40 eV): $[M+Na]^+$ 839.

3.5. (*cis*)- and (*trans*)-2-(4-{2-[(ethoxycarbonyl)amino]ethoxy} benzyl)cyclohexyl (3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oate (6a and 6b)

6a: (3α,5β,7α,12α)-3,7,12-tris(Formyloxy)cholan-24-oic acid (**5**; 160 mg, 0.325 mmol), prepared according to Maitra,[32] was dissolved in dry benzene (4 mL), the solution was cooled down in an external ice bath and then oxalyl chloride (120 μL) was added dropwise. After 45 min of stirring, volatile compounds were evaporated under reduced pressure. The crude product was dissolved in benzene (1 mL) and added to the cooled solution (0 °C) of **3a** (100 mg, 0.311 mmol) in dry benzene (1 mL) and dry pyridine (245 μL). The resulting mixture was stirred for 24 h at room temperature then was poured onto ice (30 g) and acidified by concd hydrochloric acid solution. After the extraction of aqueous phase by diethyl ether (5 × 30 mL), the crude product was purified on a silica gel column with chloroform/diethyl ether (1:1) as an eluent to give the compound **6a** (yield 87%). ^1H NMR (600 MHz, $CDCl_3$) δ 0.77 (s, 3H, H-18), 0.89 (d, 3H, J = 6.5 Hz, H-21), 0.95 (s, 3H, H-19), 1.24 (t, 3H, J = 7.2 Hz, H-16″), 2.37 (dd, J = 7.5, 13.6 Hz) + 2.39 (dd, J = 7.6, 13.8 Hz) + 2.48 (dd, J = 7.9, 13.6 Hz) + 2.65 (dd, 1H, J = 7.6, 13.8 Hz, H-7″), 3.57 (m, 2H, H-13″), 4.00 (t, 2H, J = 5.1 Hz, H-12″), 4.12 (q, 2H, J = 7.2 Hz, H-15″), 4.72 (br tt, 1H, J = 4.5, 4.5, 11.2, 11.2 Hz, H-3), 4.90 (m, 1H, H-2″), 5.08 (br s, 1H, H-7), 5.28 (br t, 1H, J = 2.9 Hz, H-12), 6.79 (m) + 6.81 (m, 2H, H-10″), 6.99 (m) + 7.10 (m, 2H, H-9″), 8.03 (d, 1H, J = 1.0 Hz, –OCOH(C-3)), 8.09 (br s) + 8.11 (br s, 1H, –OCOH(C-7)), 8.18 (br s, 1H, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.15 (q, C-18), 14.61 (q, C-16″), 17.42 + 17.46 (q, C-21), 20.84 (t, C-4″), 22.33 (t, C-15), 22.78 (q, C-19), 24.92 + 24.98 (t, C-5″), 25.53 + 25.58 (t, C-11), 26.30 + 26.55 (t, C-2), 26.94 (t, C-6″), 27.20 (t, C-16), 28.53 (d, C-9), 29.89 + 29.94 (t, C-3″), 30.91 (t, C-23), 30.98 (t, C-22), 31.32 (t, C-6), 31.59 (s, C-10), 34.26 (d, C-20), 34.41 + 34.49 (t, C-1), 34.76 + 34.78 (t, C-4), 37.69 (d, C-8), 37.72 (t, C-7″), 40.56 (t, C-13″), 40.76 (d, C-5), 42.49 (d, C-1″), 42.96 (d, C-14), 45.00 (s, C-13), 47.32 + 47.35 (d, C-17), 60.90 (t, C-15″), 66.92 (t, C-12″), 70.65 (d, C-7), 71.89 (d, C-2″), 73.72 (d, C-3), 75.29 (d, C-12), 114.16 + 114,22 (d, C-10″), 129.94 + 130.07 (d, C-9″), 132.93 (s, C-8″), 156.65 (s, C-14″), 156.72 (s, C-11″), 160.53 + 160.58 + 160.58 (d, –OCOH), 173.45 + 173.51 (s, C-24). IR (KBr): 3396, 1721, 1514, 1380, 1244, 1180 cm^{-1}. MS (ESI, 40 eV): $[M+Na]^+$ 818.

6b: *N*,*N*′-Dicyclohexylcarbodiimide (67 mg, 0.325 mmol) and 4-dimethylaminopyridine (12 mg, 0.098 mmol) were added to the mixture of 3α,5β,7α,12α-tris(formyloxy)cholan-24-oic acid (**5**; 160 mg, 0.325 mmol) and **3b** (100 mg, 0.311 mmol) in dry benzene (6 mL). The resulting mixture was stirred for 2 days at room temperature then was poured onto ice (30 g) and washed with chloroform. The aqueous phase was extracted with chloroform (5 × 30 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with chloroform/diethyl ether (1:1) as an eluent to afford **6b** (yield 77%). ^1H NMR (600 MHz, $CDCl_3$) δ 0.74 (s) + 0.75 (s, 3H, H-18), 0.85 (d, 3H, J = 6.6 Hz, H-21), 0.94 (s, 3H, H-19), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.18 (dd, J = 9.5, 13.7 Hz) + 2.81 (dd, J = 3.8, 13.7 Hz) + 2.83 (dd, 1H, J = 3.8, 13.7 Hz, H-7″), 3.58 (br q, J = 5.14 Hz, 2H, H-13″), 4.01 (t, 2H, J = 5.0 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.55 (dt, 1H, J = 4.4, 9.8, 9.8 Hz, H-2″), 4.72 (br tt, 1H, J = 4.1, 4.1, 11.4, 11.4 Hz, H-3), 5.07 (br q, 1H, J = 3.1 Hz, H-7), 5.27 (br t, 1H, J = 3.0 Hz, H-12), 6.80 (m, 2H, H-9″), 7.02 (m, 2H, H-10″), 8.03 (d, 1H, J = 0.9 Hz, –OCOH(C-3)), 8.10 (br s, 1H, –OCOH(C-7)), 8.16 (t, J = 0.8 Hz) + 8.17 (t, 1H, J = 0.8 Hz, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.12 (q, C-18), 14.62 (q, C-16″), 17.42 + 17.46 (q, C-21), 22.33 (t, C-15), 22.76 (q, C-19), 24.50 (t, C-4″), 25.04 (t, C-5″), 25.53 + 25.58 (t, C-11), 26.55 (t, C-2), 27.18 + 27.21 (t, C-16), 28.52 (d, C-9), 29.68 (t, C-6″), 29.88 (t, C-3″), 30.77 + 30.82 (t, C-23), 31.31 (t, C-6), 31.43 + 31.46 (t, C-22), 31.59 (s, C-10), 34.26 (d, C-20), 34.41 + 34.48 (t, C-1), 34.74 + 34.79 (t, C-4), 37.68 (d, C-8), 37.82 (t, C-7″), 40.46 (t, C-13″), 40.76 (d, C-5), 42.49 (d, C-1″), 43.81 + 43.84 (d, C-14), 44.97 (s, C-13), 47.25 (d, C-17), 60.91 (t, C-15″), 66.94 (t, C-12″), 70.66 (d, C-7), 73.72 (d, C-3), 75.26 (d, C-12), 76.63 (d, C-2″), 114.12 (d, C-10″), 130.10 + 130.11 (d, C-9″), 132.70 + 132.72 (s, C-8″), 156.66 (s, C-14″), 156.68 (s, C-11″), 160.53 + 160.58 + 160.59 (d, –OCOH), 173.67 + 173.73 (s, C-24). IR (KBr): 3398, 1726, 1719, 1511, 1381, 1243, 1179 cm^{-1}. MS (ESI, 40 eV): $[M+Na]^+$ 818.

3.6. (*cis*)- and (*trans*)-2-(4-{2-[(ethoxycarbonyl)amino]ethoxy} benzyl)cyclohexyl (3α,5β,7α,12α)-7,12-bis(formyloxy)-3-hydroxycholan-24-oate (7a and 7b)

Sodium bicarbonate (0.357 mmol) was added to a solution of **6a** or **6b** (0.155 mmol) in methanol (10 mL). The resulting mixture was stirred for 19 h at room temperature and then the solvent was removed. Residues were dissolved in diethyl ether, washed with a saturated aqueous solution of sodium chloride and extracted with diethyl ether (5 × 20 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with chloroform/diethyl ether (3:1 → 1:1) as an eluent to give **7a** (yield 65%) or **7b** (yield 74%). **7a**: ^1H NMR (600 MHz, $CDCl_3$) δ 0.76 (s, 3H, H-18), 0.89 (d, 3H, J = 6.6 Hz, H-21), 0.93 (s, 3H, H-19), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.37 (dd, J = 7.9, 13.7 Hz) + 2.39 (dd, J = 7.8, 13.7 Hz) + 2.54 (dd, 1H, J = 6.8, 13.7 Hz, H-7″), 3.51 (tt, 1H, J = 4.5, 4.5, 11.1, 11.1 Hz, H-3), 3.57 (br q, 2H, J = 5.2 Hz, H-13″), 4.00 (t, 2H, J = 5.1 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″),

4.90 (m, 1H, H-2″), 5.07 (br q, 1H, J = 3.2 Hz, H-7), 5.28 (t, 1H, J = 3.1 Hz, H-12), 6.79 (m, 2H, H-10″), 6.99 (m, 2H, H-9″), 8.08 (q, J = 0.9 Hz) + 8.10 (q, J = 0.9 Hz, 1H, –OCOH(C-7)), 8.16 (br t, J = 0.8 Hz, 1H, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.14 (q, C-18), 14.61 (q, C-16″), 17.42 + 17.46 (q, C-21), 20.84 (t, C-4″), 22.35 (t, C-19), 22.78 (q, C-15), 24.98 (t, C-5″), 25.50 (t, C-11), 26.93 + 26.95 (t, C-6″), 27.21 (t, C-16), 28.54 (d, C-9), 29.89 + 29.94 (t, C-3″), 30.32 (t, C-2), 30.91 + 30.97 (t, C-22), 31.45 (t, C-6), 31.53 + 31.57 (t, C-23), 34.23 (s, C-10), 34.75 + 34.77 (d, C-20), 34.84 (t, C-4), 37.72 (d, C-8), 37.72 (t, C-7″), 38.49 (t, C-1), 40.45 (t, C-13″), 40.93 (d, C-5), 42.49 (d, 1″), 42.94 (d, C-14), 44.98 (s, C-13), 47.25 + 47.30 (d, C-17), 60.90 (t, C-15″), 66.92 (t, C-12″), 70.81 (d, C-7), 71.50 (d, C-3), 71.88 (d, C-2″), 75.31 (d, C-12), 114.21 (d, C-10″), 129.94 (d, C-9″), 132.95 (s, C-8″), 156.65 (s, C-14″), 156.70 (s, C-11″), 160.58 + 160.75 (d, –OCOH), 173.47 + 173.53 (s, C-24). IR (KBr): 3409, 1719, 1512, 1382, 1244, 1179 cm^{-1}. MS (ESI, 40 eV): [M+Na]$^+$ 791. **7b**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.74 (s, 3H, H-18), 0.85 (d, 3H, J = 6.5 Hz, H-21), 0.92 (s, 3H, H-19), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.18 (dd, J = 9.5, 13.6 Hz) + 2.82 (dd, J = 3.8, 13.6 Hz) + 2.84 (dd, 1H, J = 3.8, 13.6 Hz, H-7″), 3.50 (tt, 1H, J = 4.3, 4.3, 11.2, 11.2 Hz, H-3), 3.58 (br q, J = 5.14 Hz, 2H, H-13″), 4.01 (t, 2H, J = 5.1 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.55 (dt, 1H, J = 4.2, 9.8, 9.8 Hz, H-2″), 5.06 (br q, 1H, J = 3.0 Hz, H-7), 5.26 (br t, 1H, J = 3.1 Hz, H-12), 6.80 (m, 2H, H-9″), 7.02 (m, 2H, H-10″), 8.08 (br s) + 8.10 (br s, 1H, –OCOH(C-7)), 8.13 (br s) + 8.14 (br s, 1H, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.10 (q, C-18), 14.61 (q, C-16″), 17.42 + 17.46 (q, C-21), 22.35 (t, C-19), 22.75 (q, C-15), 24.49 (t, C-4″), 25.05 (t, C-5″), 25.49 (t, C-11), 27.18 + 27.21 (t, C-16), 28.54 (d, C-9), 29.92 (t, C-6″), 30.33 (t, C-2), 30.78 (t, C-3″), 31.32 (t, C-22), 31.41 (t, C-23), 31.44 (t, C-6), 34.23 (s, C-10), 34.70 + 34.76 (d, C-20), 34.84 (t, C-4), 37.71 (d, C-8), 37.86 (t, C-7″), 38.51 (t, C-1), 40.45 (t, C-13″), 40.94 (d, C-5), 42.94 (d, C-14), 43.79 + 43.83 (d, C-1″), 44.95 (s, C-13), 47.14 + 47.18 (d, C-17), 60.91 (t, C-15″), 66.92 (t, C-12″), 70.81 (d, C-7), 71.50 (d, C-3), 75.29 (d, C-12), 76.60 + 76.63 (d, C-2″), 114.11 (d, C-10″), 130.09 (d, C-9″), 132.74 (s, C-8″), 156.65 (s, C-14″), 156.66 (s, C-11″), 160.57 + 160.73 (d, –OCOH), 173.69 + 173.75 (s, C-24). IR (KBr): 3409, 1719, 1512, 1382, 1244, 1178 cm^{-1}. MS (ESI, 40 eV): [M+Na]$^+$ 791.

3.7. (*cis*)- and (*trans*)-(3α,5β,7α,12α)-24-{[2-(4-{2-[(ethoxycarbonyl)amino]ethoxy}benzyl)cyclohexyl]oxy}-7,12-bis(formyloxy)-24-oxocholan-3-yl-(3β,22*E*)-stigmasta-5,22-dien-3-yl butanedioate (8a and 8b)

N,*N*′-Dicyclohexylcarbodiimide (0.049 mmol) and 4-pyrrolidinopyridine (0.014 mmol) were added to a mixture of **7a** or **7b** (0.052 mmol) and **2** (0.041 mmol) in dry benzene (2 mL). The resulting mixture was stirred for 2 days at room temperature then the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with petroleum ether/diethyl ether (3:2 → 1:5) as an eluent to give **8a** (yield 81%) or **8b** (yield 79%). **8a**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-18′), 0.74 (s, 3H, H-18), 0.79 (d, 3H, J = 6.6 Hz, H-27′), 0.80 (t, 3H, J = 7.3 Hz, H-29′), 0.85 (d, 3H, J = 6.6 Hz, H-21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.93 (s, 3H, H-19), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 1.26 (t, 3H, J = 7.1 Hz, H-16″), 2.37 (dd, J = 7.8, 13.7 Hz) + 2.39 (dd, J = 7.8, 13.7 Hz) + 2.54 (dd, J = 6.7, 13.7 Hz, H-7″), 3.57 (br q, 2H, J = 5.2 Hz, H-13″), 4.00 (t, 2H, J = 5.0 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.62 (tt, 1H, J = 4.5, 4.5, 11.2, 11.2 Hz, H-3), 4.62 (dddd, 1H, J = 4.1, 7.2, 9.2, 11.5 Hz, H-3′), 4.90 (dt, 1H, J = 2.5, 2.5, 4.2 Hz, H-2″), 5.01 (dd, 1H, J = 8.8, 15.1 Hz, H-23′), 5.07 (br t, 1H, J = 3.0 Hz, H-7), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.29 (br t, 1H, J = 3.1 Hz, H-12), 5.37 (dq, 1H, J = 1.8, 1.8, 1.8, 5.1 Hz, H-6′), 6.99 (m, 2H, H-9″), 6.99 (m, 2H, H-10″), 8.11 (br q, 1H, J = 0.8 Hz, –OCOH(C-7)), 8.19 (br t, 1H, J = 0.8 Hz, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.16 (q, C-18), 12.25 (q, C-29′), 14.61 (q, C-16″), 17.43 + 17.47 (q, C-21), 18.95 (q, C-27′), 19.29 (q, C-19′), 20.84 (t, C-4″), 20.97 (C-11′), 21.09 (q, C-26′), 21.20 (q, C-21′), 21.22 (t, C-6″), 22.37 (t, C-19), 22.79 (q, C-15), 24.33 (t, C-15′), 24.92 (t, C-5″), 25.40 (t, C-28′), 25.58 (t, C-11), 26.62 (t, C-2), 26.94 (t, C-16), 27.72 (t, C-2′), 28.56 (d, C-9), 28.90 (t, C-16′), 29.48 (t, C-31′), 29.48 (t, C-32′), 29.90 + 29.94 (t, C-3″), 30.92 (d, C-24′), 31.36 (t, C-6), 31.63 (t, C-22), 31.63 (t, C-7′), 31.80 (d, C-8′), 31.86 (t, C-23), 31.86 (d, C-25′), 33.93 (s, C-10), 34.30 (t, C-1), 34.57 (t, C-4), 34.77 + 34.79 (d, C-20), 36.56 (s, C-10′), 36.91 (t, C-1′), 37.67 (t, C-7″), 37.70 (d, C-8), 38.03 (t, C-4′), 39.57 (t, C-12′), 40.46 (t, C-13″), 40.50 (d, C-20′), 40.81 (d, C-5), 42.16 (s, C-13′), 42.50 (d, C-1″), 42.98 (d, C-14), 45.01 (s, C-13), 47.37 + 47.40 (d, C-17), 49.97 (d, C-9′), 55.87 (d, C-17′), 56.73 (d, C-14′), 60.91 (t, C-15″), 66.92 (t, C-12″), 70.66 (d, C-7), 71.86 (d, C-2″), 74.16 (d, C-3), 74.31 (d, C-3′), 75.28 (d, C-12), 114,22 (d, C-10″), 122.72 (d, C-6′), 129.22 (d, C-9″), 129.22 (d, C-23′), 132.94 (s, C-8″), 138.31 (d, C-22′), 139.50 (s, C-5′), 156.66 (s, C-11″), 156.74 (s, C-14″), 160.34 + 160.54 (d, –OCOH), 171.70 (s, C-30′), 171.82 (s, C-33′), 173.45 + 173.51 (s, C-24). IR (KBr): 3328, 1723, 1627, 1576, 1244, 1176 cm^{-1}. MS (ESI, 60 eV): [M+Na]$^+$ 1285. **8b**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-18′), 0.74 (s, 3H, H-18), 0.80 (d, 3H, J = 6.6 Hz, H-27′), 0.81 (t, 3H, J = 7.3 Hz, H-29′), 0.85 (d, 3H, J = 6.6 Hz, H-21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.93 (s, 3H, H-19), 1.01 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 1.25 (t, 3H, J = 7.2 Hz, H-16″), 2.18 (dd, J = 9.5, 13.7 Hz) + 2.82 (dd, J = 3.8, 13.6 Hz) + 2.83 (dd, J = 3.8, 13.6 Hz, H-7″), 3.58 (br q, 2H, J = 5.2 Hz, H-13″), 4.00 (t, 2H, J = 5.3 Hz, H-12″), 4.12 (q, 2H, J = 7.2 Hz, H-15″), 4.55 (dt, 1H, J = 4.2, 9.8, 9.8 Hz, H-2″), 4.60 (tt, 1H, J = 4.5, 4.5, 11.4, 11.4 Hz, H-3), 4.60 (dddd, 1H, J = 4.2, 7.2, 9.4, 11.1 Hz, H-3′), 5.01 (dd, 1H, J = 8.8, 15.1 Hz, H-23′), 5.07 (br t, 1H, J = 3.1 Hz, H-7), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.27 (br t, 1H, J = 3.2 Hz, H-12), 5.37 (dq, 1H, J = 1.8, 1.8, 1.8, 5.1 Hz, H-6′), 6.80 (m, 2H, H-9″), 7.02 (m, 2H, H-10″), 8.10 (br s, 1H, –OCOH(C-7)), 8.16 (br s, 1H, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.11 (q, C-18), 12.25 (q, C-29′), 14.62 (q, C-16″), 17.43 + 17.47 (q, C-21), 18.96 (q, C-27′), 19.29 (q, C-19′), 20.98 (C-11′), 21.09 (q, C-26′), 21.20 (q, C-21′), 22.37 (t, C-15), 22.76 (q, C-19), 24.33 (t, C-15′), 24.50 (t, C-4″), 24.91 (t, C-5″), 25.40 (t, C-28′), 25.58 (t, C-11), 26.61 (t, C-2), 27.19 (t, C-16), 27.72 (t, C-2′), 28.55 (d, C-9), 28.91 (t, C-16′), 29.48 (t, C-31′), 29.48 (t, C-3″), 29.69 (t, C-32′), 29.88 (t, C-6″), 30.79 (t, C-6), 30.84 (d, C-24′), 31.36 (t, C-23), 31.36 (t, C-7′), 31.51 (s, C-10), 31.81 (d, C-8′), 31.87 (t, C-22), 31.87 (d, C-25′), 34.30 (t, C-1), 34.57 (t, C-4), 34.75 + 34.81 (d, C-20), 36.57 (s, C-10′), 36.91 (t, C-1′), 37.69 (d, C-8), 37.82 (t, C-7″), 38.04 (t, C-4′), 39.58 (t, C-12′), 40.47 (t, C-13″), 40.51 (d, C-20′), 40.81 (d, C-5), 42.17 (s, C-13′), 42.97 (d, C-14), 43.81 + 43.85 (d, C-1″), 44.99 (s, C-13), 47.31 (d, C-17), 49.97 (d, C-9′), 55.87 (d, C-17′), 56.74 (d, C-14′), 60.92 (t, C-15″), 66.95 (t, C-12″), 70.67 (d, C-7), 74.16 (d, C-3), 74.31 (d, C-3′), 75.27 (d, C-12), 76.63 (d, C-2″), 114.13 (d, C-10″), 122.73 (d, C-6′), 129.22 (d, C-23′), 130.11 (d, C-9″), 132.73 (s, C-8″), 138.31 (d, C-22′), 139.50 (s, C-5′), 156.69 (s, C-11″), 156.74 (s, C-14″), 160.55 + 160.65 (d, –OCOH), 171.71 (s, C-30′), 171.83 (s, C-33′), 173.68 + 173.73 (s, C-24). IR (KBr): 3327, 1725, 1626, 1576, 1244, 1181 cm^{-1}. MS (ESI, 60 eV): [M+Na]$^+$ 1285.

3.8. (3β,22*E*)-Stigmasta-5,22-dien-3-yl-(3α,5β,7α,12α)-3,7,12-tris(formyloxy)cholan-24-oate (9)

(3α,5β,7α,12α)-3,7,12-tris(Formyloxy)cholan-24-oic acid (**5**; 1.0 g, 2.030 mmol), prepared according to Maitra,[32] was added to a mixture of stigmasterol (**1**; 0.8 g, 1.939 mmol), *N*,*N*′-dicyclohexylcarbodiimide (0.5 g, 2.423 mmol) and 4-dimethylaminopyridine

(0.07 g, 0.473 mmol) in dry benzene (10 mL). The resulting mixture was stirred for 3 days at room temperature, then concentrated under reduced pressure, diluted with water and extracted with diethyl ether (5 × 30 mL). The organic layer was dried over sodium sulfate and concentrated under reduce pressure. The crude product was purified on a silica gel column with petroleum ether/diethyl ether (4:1 → 1:1) as an eluent to give **9** as a white solid (yield 65%): mp 113–115 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.70 (s, 3H, H-18′), 0.76 (s, 3H, H-18), 0.80 (d, 3H, J = 6.5 Hz, H-27′), 0.81 (t, 3H, J = 7.3 Hz, H-29′), 0.84 (d, 3H, J = 6.6 Hz, H 21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.95 (s, 3H, H-19), 1.00 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 4.61 (dddd, 1H, J = 4.0, 7.1, 9.5, 11.5 Hz, H-3′), 4.72 (tt, 1H, J = 4.5, 4.5, 11.3, 11.3 Hz, H-3), 5.02 (dd, 1H, J = 9.3, 15.1 Hz, H-23′), 5.07 (br q, 1H, J = 3.1 Hz, H-7), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.27 (br t, 1H, J = 3.0 Hz, H-12), 5.37 (dq, 1H, J = 1.6, 1.6, 1.6, 5.1 Hz, H-6′), 8.03 (br q, 1H, J = 0.9 Hz, –OCOH(C-3)), 8.10 (d, 1H, J = 1.0 Hz, –OCOH(C-12)), 8.16 (br t, 1H, J = 0.8 Hz, –OCOH(C-7)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.14 (q, C-18), 12.25 (q, C-29′), 17.47 (q, C-21), 18.96 (q, C-27′), 19.30 (q, C-19′), 20.98 (t, C-11′), 21.10 (q, C-26′), 21.21 (q, C-21′), 22.33 (q, C-19), 22.77 (t, C-15), 24.33 (t, C-15′), 25.40 (t, C-28′), 25.53 (t, C-11), 26.56 (t, C-2), 27.18 (t, C-16), 27.77 (t, C-2′), 28.54 (d, C-9), 28.91 (t, C-16′), 30.68 (t, C-22), 31.32 (t, C-6), 31.44 (t, C-23), 31.82 (t, C-7′), 31.87 (d, C-25′), 31.87 (d, C-8′), 34.27 (s, C-10), 34.41 (t, C-4), 34.49 (t, C-1), 34.71 (d, C-20), 36.58 (s, C-10′), 36.95 (t, C-1′), 37.69 (d, C-8), 38.13 (t, C-4′), 39.58 (t, C-12′), 40.51 (d, C-20′), 40.77 (d, C-5), 42.17 (s, C-13′), 42.96 (d, C-14), 44.98 (s, C-13), 47.22 (d, C-17), 49.99 (d, C-9′), 51.21 (d, C-24′), 55.88 (d, C-17′), 56.74 (t, C-14′), 70.67 (d, C-7), 73.73 (d, C-3), 73.78 (d, C-3′), 75.28 (d, C-12), 122.62 (d, C-6′), 129.24 (d, C-23′), 138.31 (d, C-22′), 138.61 (s, C-5′), 160.52 (d, –OCOH), 160.54 (d, –OCOH), 160.58 (d, –OCOH), 173.43 (s, C-24). IR (KBr): 1723, 1374, 1185, 1175 cm^{-1}. Anal. Calcd $C_{56}H_{86}O_8$: C, 75.80; H, 10.79. Found: C, 75.43; H, 10.99. MS (ESI, 40 eV): $[M+Na]^+$ 909.

3.9. (3β,22*E*)-Stigmasta-5,22-dien-3-yl-(3α,5β,7α,12α)-7,12-bis(formyloxy)-3-hydroxycholan-24-oate (10)

Sodium bicarbonate (30 mg, 0,357 mmol) was added to a solution of **9** (150 mg, 0.169 mmol) in methanol (6 mL). The resulting mixture was stirred 5 h at room temperature then was filtered and the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with chloroform/diethyl ether (1:1) as an eluent to give **10** as a white solid (yield 78%): mp 113–116 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.70 (s, 3H, H-18′), 0.75 (s, 3H, H-18), 0.79 (d, 3H, J = 6.9 Hz, H-27′), 0.80 (t, 3H, J = 7.4 Hz, H-29′), 0.84 (d, 3H, J = 6.5 Hz, H-21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.92 (s, 3H, H-19), 1.02 (d, 3H, J = 6.5 Hz, H-21′), 1.02 (s, 3H, H-19′), 3.51 (tq, 1H, J = 4.1, 4.1, 4.1, 11.0, 11.0 Hz, H-3), 4.60 (dddd, 1H, J = 4.2, 7.7, 9.4, 11.3 Hz, H-3′), 5.02 (dd, 1H, J = 8.8, 15.2 Hz, H-23′), 5.06 (br q, 1H, J = 3.1 Hz, H-7), 5.15 (dd, 1H, J = 8.7, 15.2 Hz, H-22′), 5.27 (br t, 1H, J = 3.2 Hz, H-12), 5.37 (dq, 1H, J = 1.7, 1.7, 1.7, 5.2 Hz, H-6′), 8.10 (br q, 1H, J = 0.9 Hz, –OCOH(C-12)), 8.14 (br t, 1H, J = 0.8 Hz, –OCOH(C-7)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.12 (q, C-18), 12.25 (q, C-29′), 17.47 (q, C-21), 18.96 (q, C-27′), 19.30 (q, C-19′), 20.98 (t, C-11′), 21.09 (q, C-26′), 21.20 (q, C-21′), 22.36 (q, C-19), 22.77 (t, C-15), 24.33 (t, C-15′), 25.40 (t, C-28′), 25.49 (t, C-11), 27.18 (t, C-16), 27.77 (t, C-2′), 28.54 (d, C-9), 28.91 (t, C-16′), 30.33 (t, C-2), 30.68 (t, C-22), 31.41 (t, C-6), 31.45 (t, C-23), 31.82 (t, C-7′), 31.87 (d, C-25′), 31.87 (d, C-8′), 34.24 (s, C-10), 34.69 (d, C-20), 34.84 (t, C-4), 36.58 (s, C-10′), 36.95 (t, C-1′), 37.72 (d, C 8), 38.13 (t, C-4′), 38.50 (t, C-1), 39.58 (t, C-12′), 40.50 (d, C-20′), 40.94 (d, C-5), 42.17 (s, C-13′), 42.94 (d, C-14), 44.96 (s, C-13), 47.18 (d, C-17), 49.99 (d, C-9′), 51.21 (d, C-24′), 55.88 (d, C-17′), 56.74 (t, C-14′), 70.82 (d, C-7), 71.52 (d, C-3), 73.76 (d, C-3′), 75.29 (t, C-12), 122.61 (d, C-6′), 129.23 (d, C-23′), 138.31 (d, C-22′), 139.62 (d, C-5′), 160.58 (d, –OCOH), 160.74 (d, –OCOH), 173.45 (s, C-24). IR (KBr): 3424, 1721, 1381, 1178 cm^{-1}. Anal. Calcd $C_{55}H_{86}O_7$: C, 76.88; H, 10.09. Found: C, 76.27; H, 10.21. MS (ESI, 40 eV): $[M+Na]^+$ 881.

3.10. 4-{[(3α,5β,7α,12α)-7,12-bis(formyloxy)-24-oxo-24-[(3β,22*E*)-stigmasta-5,22-dien-3-yloxy]cholan-3-yl]oxy}-4-oxobutanoic acid (11)

4-Dimethylaminopyridine (2 mg, 0.087 mmol) was added to a solution of **10** (75 mg, 0.087 mmol) and succinic anhydride (14 mg, 0.140 mmol) in dry pyridine (2 mL). The mixture was refluxed for 4 days, then poured onto ice (20 g) and acidified by concd hydrochloric acid solution. The aqueous phase was extracted with chloroform (5 × 15 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with chloroform/methanol (80:1 → 40:1) as an eluent to give **11** as a white solid (yield 72%): mp 169–173 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.70 (s, 3H, H-18′), 0.75 (s, 3H, H-18), 0.80 (d, 3H, J = 6.5 Hz, H-27′), 0.81 (t, 3H, J = 7.3 Hz, H-29′), 0.84 (d, 3H, J = 6.6 Hz, H-26′), 0.85 (d, 3H, J = 6.5 Hz, H-21), 0.93 (s, 3H, H-19), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 2.61 (br t, 2H, J = 6.8 Hz, H-31′), 2.69 (br t, 2H, J = 6.8 Hz, H-32′), 4.64–4.58 (m, 1H, H-3′), 4.64–4.58 (m, 1H, H-3), 5.02 (dd, 1H, J = 8.9, 15.1 Hz, H-23′), 5.06 (br q, 1H, J = 3.0 Hz, H-7), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.27 (br t, 1H, J = 3.1 Hz, H-12), 5.37 (dq, 1H, J = 1.8, 1.8, 1.8, 5.1 Hz, H-6′), 8.12 (br s, 1H, –OCOH(C-7)), 8.17 (br s, 1H, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.13 (q, C-18), 12.25 (q, C-29′), 17.47 (q, C-21), 18.96 (q, C-27′), 19.30 (q, C-19′), 20.98 (t, C-11′), 21.09 (q, C-26′), 21.20 (q, C-21′), 22.35 (t, C-15), 22.77 (q, C-19), 24.33 (t, C-15′), 25.40 (t, C-28′), 25.55 (t, C-11), 26.53 (t, C-2), 27.18 (t, C-16), 27.77 (t, C-2′), 28.55 (d, C-9), 28.90 (t, C-32′), 28.90 (t, C-16′), 29.20 (t, C-31′), 30.69 (t, C-23), 31.35 (t, C-6), 31.81 (t, C-7′), 31.86 (d, C-8′), 31.86 (d, C-25′), 34.28 (d, C-20), 34.28 (t, C-4), 34.42 (t, C-1), 34.55 (s, C-10), 34.71 (t, C-22), 36.57 (s, C-10′), 36.95 (t, C-1′), 37.69 (d, C-8), 38.13 (t, C-4′), 39.58 (t, C-12′), 40.50 (d, C-20′), 40.79 (d, C-5), 42.17 (s, C-13′), 42.97 (d, C-14), 44.98 (s, C-13), 47.24 (d, C-17), 49.98 (d, C-9′), 51.20 (d, C-24′), 55.88 (d, C-17′), 56.74 (d, C-14′), 70.71 (d, C-7), 73.78 (d, C-3), 74.37 (d, C-3′), 75.29 (d, C-12), 122.62 (d, C-6′), 129.23 (d, C-23′), 138.30 (d, C-22′), 139.61 (s, C-5′), 160.62 (d, –OCOH), 160.76 (d, –OCOH), 171.67 (s, C 30′), 173.46 (s, C-24), 177.02 (s, C-33′). IR (KBr): 3435, 1722, 1383, 1177 cm^{-1}. Anal. Calcd $C_{59}H_{90}O_{10}$: C, 73.86; H, 9.46. Found: C, 74.12; H, 9.08. MS (ESI, 40 eV): $[M+Na]^+$ 982.

3.11. (*cis*)- and (*trans*)-(3α,5β,7α,12α)-7,12-bis(formyloxy)-24-oxo-24-[(3β,22*E*)-stigmasta-5,22-dien-3-yloxy]cholan-3-yl-2-{4-{2-[(ethoxycarbonyl)amino]ethoxy}benzyl}cyclohexyl butanedioate (12a and 12b)

N,*N*′-Dicyclohexylcarbodiimide (0.097 mmol) and 4-pyrrolidinopyridine (0.027 mmol) were added to a mixture of **11** (0.083 mmol) and **3a** or **3b** (0.106 mmol) in dry dichloromethane (4 mL). The resulting mixture was stirred for 2 days at room temperature then the solvent was removed under reduced pressure. The crude product was purified on a silica gel column with petroleum ether/diethyl ether (1:1 → 1:2) as an eluent to give **12a** (yield 86%) or **12b** (yield 93%). **12a**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.70 (s, 3H, H-18′), 0.75 (s, 3H, H-18), 0.79 (d, 3H, J = 6.6 Hz, H-27′), 0.81 (t, 3H, J = 7.3 Hz, H-29′), 0.84 (d, 3H, J = 6.7 Hz, H-21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.93 (s, 3H, H-19), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.7 Hz, H-21′), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.38 (dd,

J = 7.7, 13.7 Hz) + 2.39 (dd, J = 7.7, 13.7 Hz) + 2.53 (dd, J = 7.1, 13.7 Hz, H-7″), 3.57 (br q, 2H, J = 5.2 Hz, H-13″), 4.00 (t, 2H, J = 5.2 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.60 (tt, 1H, J = 4.6, 4.6, 11.3, 11.3 Hz, H-3), 4.62 (dddd, 1H, J = 4.2, 7.1, 9.2, 11.1 Hz, H-3′), 4.92 (br dt, 1H, J = 2.5, 2.5, 4.2 Hz, H-2″), 5.01 (dd, 1H, J = 8.8, 15.1 Hz, H-23′), 5.07 (br s, 1H, H-7), 5.15 (dd, 1H, J = 8.7, 15.1 Hz, H-22′), 5.27 (br t, 1H, J = 3.0 Hz, H-12), 5.37 (dq, 1H, J = 1.8, 1.8, 1.8, 5.1 Hz, H-6′), 6.79 (m, 2H, H-9″), 7.01 (m, 2H, H-10″), 8.11 (br q, J = 0.8 Hz) + 8.16 (br q, 1H, J = 0.7 Hz, –OCOH(C-7)), 8.14 (br t, J = 0.8 Hz) + 8.16 (br t, 1H, J = 0.8 Hz, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.02 (q, C-18′), 12.13 (q, C-18), 12.25 (q, C-29′), 14.61 (q, C-16″), 17.47 (q, C-21), 18.95 (q, C-27′), 19.30 (q, C-19′), 20.76 (q, C-26′), 20.76 (t, C-5″), 20.98 (C-11′), 21.20 (q, C-21′), 22.36 (t, C-15), 22.77 (q, C-19), 24.33 (t, C-15′), 25.40 (t, C-28′), 25.58 (t, C-11), 25.58 (t, C-4″), 26.63 (t, C-2), 26.95 (t, C-3″), 27.18 (t, C-16), 27.77 (t, C-2′), 28.56 (d, C-9), 28.91 (t, C-16′), 29.58 (t, C-32′), 29.68 (t, C-31′), 29.88 (t, C-6″), 30.70 (t, C-23), 31.37 (t, C-6), 31.81 (t, C-7′), 31.86 (d, C-8′), 31.86 (d, C-25′), 33.92 (d, C-20), 34.30 (t, C-4), 34.52 (s, C-10), 34.58 (t, C-1), 34.71 (t, C-22), 36.57 (s, C-10′), 36.95 (t, C-1′), 37.69 (t, C-7″), 37.69 (d, C-8), 38.13 (t, C-4′), 39.58 (t, C-12′), 40.51 (d, C-20′), 40.80 (t, C-13″), 42.16 (s, C-13′), 42.54 (d, C-1″), 42.97 (d, C-14), 44.98 (s, C-13), 47.23 (d, C-17), 49.17 (d, C-5), 49.98 (d, C-9′), 51.21 (d, C-24′), 55.87 (d, C-17′), 56.74 (d, C-14′), 60.72 (t, C-15″), 66.91 (t, C-12″), 70.68 (d, C-7), 72.39 (d, C-2″), 73.78 (d, C-3), 74.23 (d, C-3′), 75.30 (d, C-12), 114,21 (d, C-10″), 122.62 (d, C-6′), 129.23 (d, C-23′), 130.01 (d, C-9″), 132.93 (s, C-8″), 138.31 (d, C-22′), 139.61 (s, C-5′), 156.70 (s, C-11″), 160.56 (s, C-14″), 160.56 + 160.65 (d, –OCOH), 171.62 (s, C-30′), 171.83 (s, C-33′), 173.45 (s, C-24). IR (KBr): 3339, 1726, 1512, 1242, 1175 cm^{-1}. MS (ESI, 60 eV): $[M+Na]^+$ 1285. **12b**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-18′), 0.75 (s, 3H, H-18), 0.79 (d, 3H, J = 6.6 Hz, H-27′), 0.80 (t, 3H, J = 7.3 Hz, H-29′), 0.84 (d, 3H, J = 6.6 Hz, H-21), 0.85 (d, 3H, J = 6.5 Hz, H-26′), 0.93 (s, 3H, H-19), 1.02 (s, 3H, H-19′), 1.02 (d, 3H, J = 6.6 Hz, H-21′), 1.25 (t, 3H, J = 7.1 Hz, H-16″), 2.20 (dd, J = 9.2, 13.8 Hz) + 2.82 (dd, J = 3.8, 13.8 Hz, H-7″), 3.57 (br q, 2H, J = 5.4 Hz, H-13″), 4.01 (t, 2H, J = 5.1 Hz, H-12″), 4.12 (q, 2H, J = 7.1 Hz, H-15″), 4.57 (m, 1H, H-2″), 4.60 (tt, 1H, J = 4.6, 4.6, 11.4, 11.4 Hz, H-3), 4.60 (dddd, 1H, J = 4.1, 7.3, 9.5, 11.3 Hz, H-3′), 5.01 (dd, 1H, J = 8.9, 15.2 Hz, H-23′), 5.06 (br s, 1H, H-7), 5.15 (dd, 1H, J = 8.7, 15.2 Hz, H-22′), 5.27 (br t, 1H, J = 3.0 Hz, H-12), 5.37 (dq, 1H, J = 1.8, 1.8, 1.8, 5.1 Hz, H-6′), 6.80 (m, 2H, H-9″), 7.02 (m, 2H, H-10″), 8.09 (br q, J = 0.8 Hz) + 8.11 (br q, 1H, J = 0.7 Hz, –OCOH(C-7)), 8.14 (br t, J = 0.8 Hz) + 8.16 (br t, 1H, J = 0.8 Hz, –OCOH(C-12)). ^{13}C NMR (150 MHz, $CDCl_3$) δ 12.03 (q, C-18′), 12.13 (q, C-18), 12.25 (q, C-29′), 14.63 (q, C-16″), 17.48 (q, C-21), 18.96 (q, C-27′), 19.31 (q, C-19′), 20.99 (C-11′), 21.09 (q, C-26′), 21.21 (q, C-21′), 22.36 (t, C-15), 22.78 (q, C-19), 24.34 (t, C-15′), 24.50 (t, C-4″), 24.91 (t, C-5″), 25.41 (t, C-28′), 25.59 (t, C-11), 26.62 (t, C-2), 27.19 (t, C-16), 27.78 (t, C-2′), 28.56 (d, C-9), 28.91 (t, C-16′), 29.46 (t, C-32′), 29.49 (t, C-31′), 29.89 (t, C-6″), 30.70 (t, C-23), 31.35 (t, C-6), 31.78 + 31.82 (t, C-3″), 31.82 (t, C-7′), 31.87 (d, C-8′), 31.87 (d, C-25′), 33.93 (d, C-20), 34.29 (t, C-4), 34.50 (s, C-10), 34.56 (t, C-1), 34.72 (t, C-22), 36.58 (s, C-10′), 36.96 (t, C-1′), 37.69 (d, C-8), 37.79 (t, C-7″), 38.13 (t, C-4′), 39.59 (t, C-12′), 40.51 (t, C-13″), 40.51 (d, C-20′), 40.81 (d, C-5), 42.17 (s, C-13′), 42.98 (d, C-14), 43.81 (d, C-1″), 44.99 (s, C-13), 47.25 (d, C-17), 49.99 (d, C-9′), 51.21 (d, C-24′), 55.88 (d, C-17′), 56.75 (d, C-14′), 60.91 (t, C-15″), 66.94 (t, C-12″), 70.69 (d, C-7), 73.78 (d, C-3), 74.18 (d, C-3′), 75.28 (d, C-12), 76.63 (d, C-2″), 114,11 (d, C-10″), 122.62 (d, C-6′), 129.24 (d, C-23′), 130.17 (d, C-9″), 132.71 (s, C-8″), 138.31 (d, C-22′), 139.61 (s, C-5′), 156.68 (s, C-11″), 156.68 (s, C-14″), 160.55 + 160.63 (d, –OCOH), 171.80 (s, C-30′), 171.95 (s, C-33′), 173.44 (s, C-24). IR (KBr): 3327, 1733, 1731, 1627, 1576, 1244, 1162 cm^{-1}. MS (ESI, 60 eV): $[M+Na]^+$ 1285.

3.12. Screening tests of the selected pro-juvenoids on the red firebug (*P. apterus*)

(a) Topical screening tests: compounds to be tested were dissolved in acetone in three concentrations (0.05, 0.5 and 5 μg μL^{-1}). This solution (1 μL) was applied on the top of freshly molted nymph of the fifth instar of *P. apterus* by using Burkhard microapplicator. Acetone (1 μL) was used to treat insects in the reference experiment.

(b) Oral screening tests: application of the tested pro-juvenoids was made by a drinking assay according to an already published methodology.[34] Each tested compound was dissolved in acetone (200 μL) and a solution was added into a mixture of a distilled water (50 mL) and Tween-80 (5 μL) to give concentrations of the tested pro-juvenoid 0.025, 0.25, 2.5, and 25 μg μL^{-1}. The resulting solutions were offered in glass vials plugged with pieces of cotton at the end of the fourth nymphal instar of *P. apterus*. A mixture of acetone, distilled water, and Tween-80 was used in the reference experiment.

Each concentration of the tested compound was applied to 10 individuals and all experiments were performed in three replications. The tested insects were put into Petri dishes and they were kept in a climatic box under artificial lighting (16L:8D) at a temperature 25 ± 0.5 °C and at a relative humidity 50 ± 5%. The development and mortality of the tested insects were checked every day. The resulting biological activity was evaluated according to the degree of inhibition of metamorphosis determined by morphological changes after the last ecdysis.[8,33] The results of the screening tests are summarized in Table 1.

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