Characterization of 17β-estradiol 3-(β-*D*-glucopyranoside) and 17-(α-*D*-glucopyranoside) as the metabolites of 17β-estradiol in the cultured ovaries of the silkworm, *Bombyx mori*

Y. Fujimoto, N. Ikekawa, M. Ogiso[1] and E. Ohnishi

Dept of Chemistry, Tokyo Institute of Technology, Meguro-ku, Tokyo 152 (Japan), and Biological Institute, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464 (Japan), 23 July 1985

Summary. The structures of the metabolites formed upon incubation of 17β-estradiol with the ovaries of silkworm, *Bombyx mori*, have been determined as 17β-estradiol 3-(β-*D*-glucopyranoside) (**1**) and 17-(α-*D*-glucopyranoside) (**2**) by spectroscopic means.
Key words. Silkworm; *Bombyx mori*; 17β-estradiol metabolites; ovaries.

^{1}H NMR data (400 MHz, recorded in $CDCl_3$) of the pentaacetates (**3**) and (**4**), and 17β-estradiol diacetate[a]

Assignment	Compound (**3**)	Compound (**4**)	Estradiol diacetate
1-H	7.20 (d, 8.4)	7.28 (d, 8.5)	7.28 (d, 8.4)
2-H	6.77 (dd, 8.4, 2.6)	6.84 (dd, 8.5, 2.4)	6.84 (dd, 8.4, 2.6)
4-H	6.71 (d, 2.6)	6.79 (d, 2.4)	6.79 (d, 2.6)
17-H	4.69 (d, 9.0, 8.0)	3.64 (dd, 7.5, 8.0)	4.69, (dd, 9.0, 8.0)
3-OAc	–	2.28 (s)	2.28 (s)
17-OAc	2.06 (s)	–	2.06 (s)
18-CH_3	0.82 (s)	0.85 (s)	0.82 (s)
1′-H	5.04 (d, 7.8)	5.14 (d, 3.9)	–
2′-H	5.25 (t, 9.0)	4.80 (dd, 10.2, 3.8)	–
3′-H	5.29 (t, 9.0)	5.45 (t, 9.8)	–
4′-H	5.16 (t, 9.5)	5.04 (t, 9.7)	–
5′-H	3.85 (ddd, 10.0, 5.3, 2.5)	4.12 (m)	–
6′-Ha	4.17 (d, 12.4, 2.6)	4.12 (m)	–
6′-Hb	4.27 (d, 12.4, 5.2)	4.26 (dd, 12.9, 5.3)	–
2′-OAc	2.04 (s)	2.02 (s)	–
3′-OAc	2.05 (s)	2.05 (s)	–
4′-OAc	2.05 (s)	2.06 (s)	–
6′-OAc	2.09 (s)	2.10 (s)	–

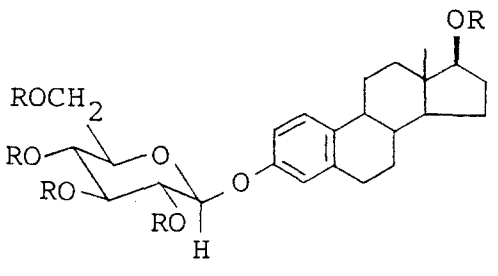
[a]Chemical shift in ppm and coupling constant in Hz.

We have recently identified estradiol in the ovaries of the silkworm, *Bombyx mori*[2]. In conjunction with our project to investigate the possible biological roles of steroid hormones in insects, we have studied the metabolism of 17β-estradiol in the cultured ovaries of *B. mori*. Preliminary labeling experiments indicated the formation of at least three polar metabolites, which were designated **A**, **B** and **C** (R_f values[3] 0.36, 0.42 and 0.01, respectively, on a precoated silica gel plate (Merck 60 F_{254}, 0.5 mm thickness) with $CHCl_3$-96% aqueous ethanol, 4:1, as the developing solvent)[4]. We have now characterized the metabolites **A** and **B** as 17β-estradiol 3-(β-*D*-glucopyranoside) (**1**) and 17-(α-*D*-glucopyranoside) (**2**), respectively.

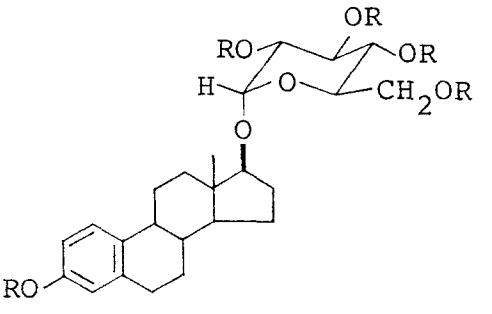
The obtain materials for structure elucidation, non-labeled substrate was incubated and metabolites were isolated according to procedures essentially the same as previously reported[5]. 17β-estradiol (100 μg) was incubated in Grace's insect medium (10 ml) in the presence of the ovarian tissues (40 heads, dissected from the pupae 60 h after larval-pupal ecdysis) of *B. mori* for 5 h. The incubation was repeated 5 times. Apolar and polar forms of steroids were separated by silicic acid column chromatography with benzene-methanol, 9:1, and methanol, respectively. For the isolation of apolar compounds (containing **A** and **B**), eluate from the silicic acid column was applied to TLC (aforementioned conditions). The bands corresponding to **A** and **B** were scraped from the TLC plate and eluted with methanol, and purified by HPLC using a reversed phase column Wakogel ODS-10K (4.0 mm i.d. × 50 cm) and solvent system of methanol-water, 3:2, flow rate being 1 ml/min. Fractions eluted at 12.2 min and 17.0 min[6] afforded the metabolite **A** (80 μg)[7], UV (MeOH) λ_{max} 278 nm, and **B** (20 μg)[7], UV (MeOH) λ_{max} 280 nm. At the outset of the work, we thought **A** and **B** might be a hydroxylated estradiol derivative such as estriol, judging from their mobility in HPLC. This, however, turned out to be incorrect after the ^{1}H NMR measurement of these metabolites.

The ^{1}H NMR data[8] (in CD_3OD) revealed that the two metabolites are mono-sugar substituted estradiols; **A** has the substitution at 3-position with 17-OH being intact; whereas **B** has the substitution at 17-position with 3-OH being intact, although the overlapped signals at δ 3.3–3.7 disturbed a detailed analysis. FAB mass spectra of (**1**) and (**2**) both exhibited a peak at *m/z* 457 [presumably M^+ ($C_{24}H_{37}O_7$)+Na]. Further, the peracetylated compounds (**3**) and (**4**), obtained from (**1**) and (**2**), respectively, by treatment with Ac_2O-pyridine at room temperature, showed a peak at *m/z* 644 (M^+, $C_{34}H_{44}O_{12}$, corresponds to the penta-acetate of (**1**) and (**2**)) in their FD mass spectra. These mass spectral data indicated that the sugar moiety is a hexose.

The analysis of the ^{1}H NMR data of the penta-acetates (**3**) and (**4**) allowed the assignation of the hexose as glucose. All the hydrogen signals of the glucose moiety were unambiguously assigned as listed in the table by the aid of decoupling experiments, and the coupling constants between adjacent hydrogens were quite reasonable as for the *J*-values of glucose (acetate). Comparison of the ^{1}H NMR. data of (**1**) and (**2**) with that of 17β-estradiol diacetate (the data is shown in the table) confirmed the position of glucose substitution. Stereochemistry of the glucoside linkage was determined as β-glucoside for (**3**) and α-glucoside for (**4**) on the basis of the observed coupling constants for



(**1**): R=H
(**3**): R=Ac



(**2**): R=H
(**4**): R=Ac

the anomeric protons (H'−1: J = 7.8 Hz for (**3**) and J = 3.9 Hz for (**4**)). 17β-stereochemistry for both (**3**) and (**4**) was deduced on the basis of the coupling patterns (triplet-like) of 17α-hydrogen signals, since it was reported that 17α-hydrogen resonance of 17β-OH derivatives is observed as nearly a triplet, but 17β-hydrogen resonance of 17α-OH derivatives as a doublet[9]. Thus, the structures of the metabolites **A** and **B** were established as those shown in the formulae (**1**) and (**2**), respectively. Enzymatic hydrolysis of the ^{14}C-labeled[10] (**1**) and (**2**) supported the assigned structures. Incubation of (**1**) with a β-glucosidase preparation from sweet almonds (Boehringer Mannheim) in 10 mM acetate buffer (pH 4.8) at 25 °C for 4 h, released 17β-estradiol while incubation of (**2**) with an α-glucosidase preparation from yeast (Boehringer Mannheim, grade I) under the same condition afforded 17β-estradiol as well, although the reaction was incomplete[10].

The present work provides the first rigorous structure characterization of steroid glucosides isolated from arthropods, although the formation of steroid glucosides in arthropods has been previously described, but without identification of the structures[11]. The two glucosides (**1**) and (**2**) are hitherto unknown metabolites of 17β-estradiol[12]. In insects the transfer of glucose from UDP-glucose to phenolic groups has been reported[13]. Glycoside synthesizing activity toward steroid hormones is known in vertebrates[14], plants[15], and microorganism[16]. The formation of the β-glucoside and α-glucoside together suggests the occurrence of both β- and α-glucosyltransferases in silkworm ovaries. It will be of great interest to see whether the glucosides exist in the ovaries of the insect.

Acknowledgment. We are grateful to Mr T. Eguchi for the measurements of ^{1}H NMR spectra.

1 Present address: Department of Physiology, Toho University School of Medicine, Ohmori-nishi, Tokyo 143, Japan.

2 Ohnishi, E., Ogiso, M., Wakabayashi, K., Ikekawa, N., and Fujimoto, Y., Gen. comp. Endocr. *60* (1985) 35.
3 The R_f value for 17β-estradiol is 0.56.
4 Detailed results of the metabolic experiments including other steroid hormones such as testosterone will be reported elsewhere. Homogeneity of the metabolite **C** was not fully investigated.
5 Ogiso, M., and Ohnishi, E., Molec. cell. Endocr. *38* (1984) 13.
6 The retention time for estriol is 9.8 min.
7 The weight was estimated on the basis of UV intensity (ε value 2000 was used for the calculation).
8 ^{1}H NMR data: (**1**) δ4.89 (d with a virtual coupling 7.2 Hz, 1'-H), 7.20 (1-H), 6.85 (2-H), 6.78 (4-H); (**2**) 4.80 (d, 3.7 Hz, 1'-H), 7.07 (1-H), 6.52 (2-H), 6.47 (4-H). For the virtual coupling, see: Dahmen, J., Fried, T., Gronberg, G., Magnusson, G., and Noori, G., Carbohydr. Res. *125* (1983) 161.
9 Williamson, D.G., Collins, D.C., Layne, D.S., Conrow, R.B., and Bernstein, S., Biochemistry *8* (1969) 4299. This reference includes the ^{1}H NMR data of 17α- and 17β-estradiol 17-(β-*D*-glucopyranosides) penta-acetates, which are different from that of (**4**).
10 For the labeled compounds and quantitative data, see reference 2.
11 E.g. Heinrich, G., and Hoffmeister, H., Z. Naturf. *25B* (1970) 358; Kaplanis, J.N., Dutky, S.R., Robbins, W.E., and Thompson, M.J., in: Invertebrate Endocrinology and Hormonal Heterophylly, p. 161. Ed. W.J. Burdette. Springer-Verlag, New York 1974; McCarthy, J.F., and Skinner, D.M., Gen. comp. Endocr. *37* (1979) 250.
12 Tentative identification of (**1**) as an incubation product of 17β-estradiol by rabbit liver glucosyltransferase, see: Collins, D.C., Williamson, D.G., and Layne, D.S., J. biol. Chem. *245* (1970) 873.
13 Brunet, P.C.J., Insect Biochem. *10* (1980) 467.
14 Lobow, R.S., Williamson, D.G., Layne, D.S., and Collins, D.C., Can. J. Biochem. *53* (1975) 1028; Williamson, D.G., Layne, D.S., and Collins, D.C., J. biol. Chem. *247* (1972) 2386.
15 Prochazka, Z., Collection Czech. chem. Commun. *33* (1968) 4039.
16 Pan, S.C., Biochemistry *9* (1970) 1833.

0014-4754/86/050567-02$1.50 + 0.20/0

The effect of the phytoalexins, lubimin, (-)-maackiain, pinosylvin, and the related compounds dehydroloroglossol and hordatine M on human lymphoblastoid cell lines[1]

L. Skinnider and A. Stoessl

Department of Pathology, University of Saskatchewan, Saskatoon (Saskatchewan, Canada S7N OWO), and Research Centre, Agriculture Canada, University Sub Post Office, London (Ontario, Canada N6A 5B7), 5 August 1985

Summary. We have tested the effect of the phytoalexins lubimin, (-)-maackiain and pinosylvin and the related compounds dehydroloroglossol and hordatine M on the growth of the human lymphoblastoid cell lines Molt and Raji. (−)-maackiain, pinosylvin and dehydroloroglossol showed significant growth inhibitory action on the cells. Suppression of [^{3}H] thymidine and [^{3}H] leucine uptake was tested and noted in pinosylvin and dehydroloroglossol. The phytoalexins and related compounds are widespread in plants and provide a potential source of antineoplastic substances.
Key words. Phytoalexins; growth inhibitory, transformed cells.

The generic term phytoalexin has been developed to describe substances newly synthesized by infected plants which possess antifungal activity against a wide range of fungi[2,3] although now this view has been somewhat modified and indeed at present there is no universally accepted definition[4]. Their production by plants is stimulated not only by the fungi themselves but also by substances from fungal culture filtrates or from extracts of fungal tissues[5,6]. These substances are called 'inducers' or 'elicitors'. The phytoalexins themselves consist of a wide variety of low molecular weight compounds with a preponderance at present of isoflavanoid and isoprenoid compounds. Their inhibitory action on fungal growth is well documented[2,7] but their effects on mammalian systems have been investigated only cursorily[8]. Studies have been published suggesting a possible antineoplastic role of the sesquiterpenoid phytoalexin capsidiol and the dihydrophenanthrene phytoalexin orchinol[9], and of the preinfectionally occurring, antimicrobial sesquiterpene parthenolide[10] as well as the terpenoid dihydrophenanthrene juncusol[11]. The last of these compounds may be a phytoalexin but this has not yet been established. This report documents the effect of other phytoalexins, viz. lubimin, (−)-maackiain, and pinosylyin, on transformed human cell lines and their normal counterparts. The synthetic compound dehydroloroglossol, closely related to the orchid phytoalexin loroglossol, and the preinfectional antifungal factor hordatine M were also tested.

Materials and methods. Test substances. Lubimin, from potatoes inoculated with *Alternaria solani,* and hordatine M, from uninfected barley seedlings, were available from other studies[12,13]. (−)-Maackiain was prepared by known procedures[14,15] from trifolirhizin tetraacetate which was a generous gift from Prof. S.