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BIOCONVERSION OF STEROID GLYCOSIDES BY *NOCARDIA RESTRICTA*

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Summary—The bioconversion of steroid alkaloid tomatine by *Nocardia restricta* yields the conjugate with lactic acid. We studied the bioconversion of some steroid glycosides without a nitrogen atom in the molecule to determine the effect of the nitrogen atom. The glycosides were of three different types: sterol glycosides, bufadienolide rhamnoside and steroid saponine. The results of bioconversions showed that *Nocardia restricta* converts steroid glycosides differently according to the sugar bound to the steroid aglycone. It can be concluded that in the absence of a nitrogen atom in the steroid molecule no conjugation with lactic acid by *Nocardia restricta* occurs.

INTRODUCTION

The bioconversion of tomatine, the glycoside of the steroid alkaloid tomatidine, is known to proceed in three different ways. It has been found by Arneson and Durbin[1] that glucose is split off from α-tomatine by *Septoria lycopersici*. It has also been reported by Ford *et al.*[2] that *Fusarium oxysporum* f.sp. *lycopersici* cleaves α-tomatine into tomatidine and the tetrasaccharide lycotetraose. Belič *et al.*[3] have found that the bioconversion of α-tomatine by *Nocardia restricta* proceeds in a different way, yielding a conjugate of α-tomatine with L(+)-lactic acid. For the first two modes of microbial bioconversions there are numerous examples described in the literature for different substrates and microbes. For the third one, however, this has only been observed with tomatine to date and it thus represents the first example of a new mode of microbial bioconversion. The question arises if this mode of bioconversion occurs also with other types of steroid glycosides or if the presence of a nitrogen atom in the molecule of the substance is a prerequisite for the conjugation of the L(+)-lactic acid to the steroid glycoside.

EXPERIMENTAL

Chemicals and reagents

Cholesterol was supplied by BDH. Proscillaridin A was obtained from B. Pelan, chem. eng., Research Department, Lek, Ljubljana. Diosgenin was purchased from Steraloids and D(+)-galactose from Merck, p.a. For column chromatography silica gel S purchased from Riedel de Haen was used.

Methods

Thin layer and column chromatography methods were used for the isolation of products of the bioconversion of steroid glycosides. Solvent systems for both methods are described in the preparation of steroid glycosides.

Molecular weights of substrates and their metabolites were determined by mass spectrometry using a CEC 21-110 C double focusing instrument. Fast atom bombardment mass spectrometric (FAB MS) analysis was performed by means of fast xenon atoms of about 5 keV energy produced by a modified electron oscillation ion source. This mode of MS analysis gave protonated molecular ions $(M + H)^+$ from which the molecular weights for the dehydrogenated products of proscillaridin A were deduced. The chemical ionization mass spectrometry with the isobutane–ammonia (1:1, v/v) reagent gas was used [6]. This analysis gave molecular ions $(M + NH_4)^+$ for β-D-cholesteryl glucoside and β-D-cholesteryl galactoside.

Preparation of steroid glycoside

β-D-cholesteryl glucoside

It was synthesized from cholesterol and tetraacetyl bromglucose using the method of Meystre and Miescher[4]. Its purity was checked by TLC on silica gel [5]. Cl mass spectrum: m/z 566 $(M + NH_4)^+$, 565 $(M + NH_3)^+$. i.r. spectrum (KBr): 3400, 2940, (C–O–C) 1030 cm^{-1}.

Hydrolysis of the metabolite of β-D-cholesteryl glucoside. The metabolite (2 mg) obtained in the bioconversion of β-D-cholesteryl glucoside and 0.3 ml of 2% methanolic KOH, was heated at 60°C for 2 h. After neutralisation with 0.2 NHCl the reaction mixture was dried and dissolved in 70% ethanol. The products of hydrolysis were analysed with paper chromatography and with TLC. The descending paper chromatography analysis was done on Whatman No. 1 in *n*-butanol–acetic acid–water solvent system (4:1:5, by vol.). TLC was done on DC-Fertigplatten G 1440/A 10 Cellulose with 10% Dowex (Selecta, Schleicher and Schüll 2-X 8) in

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butanol–acetic acid–water solvent system (60:20:20, by vol.). The presence of glucose could be excluded by the use of aniline–phthlate reagent by both methods.

β-D-cholesteryl galactoside. It was synthesized from D-tetraacetyl-cholesteryl galactoside using the method of Horlin *et al.*[9] for the saponification of β-D-tetraacetyl–cholesteryl glucoside.

To 4 mmol of sodium in 250 ml abs. methanol 1 mmol of β-D-tetraacetyl–cholesteryl galactoside was added. The reaction mixture was left standing overnight. An equal amount of water was added and the mixture extracted 3 times with butanol–toluene (1:1, v/v). The extract was washed three times with water, dried over Na_2SO_4 and analysed on silica gel G 254 in the chloroform–methanol–acetic acid solvent system (90:10:2, by vol.). After detection with 50% H_2SO_4 in ethanol the β-D-cholesteryl galactoside appeared as a red spot with R_f 0.65. It was crystalized from 70% ethanol. Cl mass spectrum: *m/z* 566 $(M+NH_4)^+$, 565 $(M+NH_3)^+$.

β-D-diosgenyl galactoside. β-D-tetraacetyl–diosgenyl galactoside was synthesized from diosgenin and tetraacetyl bromgalactose using the method of Meystre and Miescher[1]. Tetraacetyl bromgalactose was synthesized from D-galactose using the method of Bárczai–Martos *et al.*[5] for tetraacetyl bromglucose. The purity of D-tetraacetyl–diosgenyl–galactoside was checked by TLC on silica gel G in ethylacetate (**I**) and ethylacetate–cyclohexane (2:1, v/v) (**II**) solvent systems. After spraying the plate with the 50% H_2SO_4 in 90% ethanol and heating it at 120°C a red spot appeared, R_f values 0.71 in (**I**) and 0.73 in (**II**).

β-D-diosgenyl galactoside was synthesized by the saponification of β-D-tetraacetyl–diosgenyl galactoside by the method of Horlin *et al.*[6]. To 4.82 mmol of sodium in 125 ml abs. methanol 1.21 mmol of β-D-tetraacetyl–diosgenyl galactoside was added. The reaction mixture was left standing overnight. An equal amount of water was added and the mixture extracted 3 times with butanol–toluene (1:1, v/v) solvent mixture. The extract was washed with water, dried over Na_2SO_4, evaporated and analysed on silica gel G in ethylacetate–cyclohexane (2:1, v/v) (**III**) and chloroform–methanol–1% NH_4OH (2:2:1, by vol.) (**IV**) solvent systems. After spraying the plate with 50% H_2SO_4 in 90% ethanol a red–violet spot of diosgenyl galactoside appeared with the R_f values 0.0 in (**III**) and 0.8 in (**IV**) beside of unreacted diosgenin R_f 0.69 in (**III**) and 0.97 in (**IV**). The diosgenyl galactoside was isolated from the column of silica gel S. The crude mixture was applied to the column dissolved in 70% ethanol and eluted with ethylacetate–cyclohexane (2:1, v/v). FAB mass spectrum: *m/z* 577 $(M^+ + 1)$.

Bioconversions

Nocardia restricta CBS 157.45 was grown on potato–agar slants for 10 days and then transferred to 100 ml of the growth medium and shaken at 28°C and 280 r.p.m. for 3 days. The cells were centrifuged at 2000 r.p.m. for 5 min, washed with 0.4% NaCl solution, centrifuged and the cells from two flasks transferred into a flask with 100 ml of 0.4% NaCl solution.

Bioconversion of β-D-cholesteryl glucoside. Cholesteryl glucoside (10 mg) was dissolved in 1 ml of 70% ethanol, added to 100 ml of the 0.4% NaCl suspension of cells and the incubation continued for 3 days. Metabolites were extracted by chloroform and butanol, solvents evaporated and the residue analysed by TLC in chloroform–methanol–acetic acid solvent system (90:10:2, by vol.) on silica gel GF_{254}. The plates were sprayed with 50% H_2SO_4 in 90% ethanol and heated at 120°C for 10 min. The unreacted β-D-cholesteryl glucoside and the metabolite appeared as red spots with the R_f values of 0.37 and 0.72. The metabolite was isolated by TLC. i.r. spectrum (film): 2940, 2860, 1580, 1410 cm^{-1}. Mass spectrum: *m/z* 386 (M^+), 369, 368, 353, 275, 256. It was identical with the mass spectrum of cholesterol.

Bioconversion of β-D-cholesteryl galactoside. The bioconversion was done as described for β-D-cholesteryl glucoside. TLC analysis of the extract of transformation broth did not show any new metabolites.

Bioconversion of proscillaridin A. Proscillaridin A (10 mg) was dissolved in 1 ml of ethanol and added to 100 ml of the 0.4% NaCl suspension of cells. The incubation was continued for 3 days. The incubation broth was extracted by butanol. The butanol extract was analysed by TLC on silica gel GF_{254} in ethylacetate solvent. Two new metabolites with R_f 0.25 and 0.54 appeared besides the unreacted proscillaridin A, R_f 0.12. The molecular ion at *m/z* 381 $(M^+ + 1)$ in the FAB mass spectrum of the metabolite with the R_f value of 0.25 corresponds to the loss of 6 hydrogen atoms in addition to the splitting off of rhamnose from proscillaridin A. The new double bonds are located in ring A by the ion *m/z* 121 (EI mass spectrum) and by the absorption maximum in ethanol at 244 nm, in agreement with the 1,4-diene-3-keto structure. Therefore, the structure of this metabolite is 3-keto-14β-hydroxy-bufa-1,4,20,22-tetraenolide.

The molecular ion at *m/z* 383 $(M^+ + 1)$ in the FAB mass spectrum of the metabolite with the R_f value of 0.54 corresponds to the loss of four hydrogen atoms in addition to the splitting off of rhamnose from proscillaridin A. The new double bonds are located in ring A by the ion *m/z* 123 (EI mass spectrum) and by the absorption maximum in ethanol of 241 nm, in agreement with the 3-keto-4-ene structure. The structure of this metabolite is, therefore, 3-keto-14β-hydroxy-bufa-4,20,22-trienolide.

Bioconversion of β-D-diosgenyl galactoside. β-D-diosgenyl galactoside (10 mg) dissolved in 1 ml of 70% ethanol with some drops of chloroform to get a clear solution was added to 100 ml of the cell's

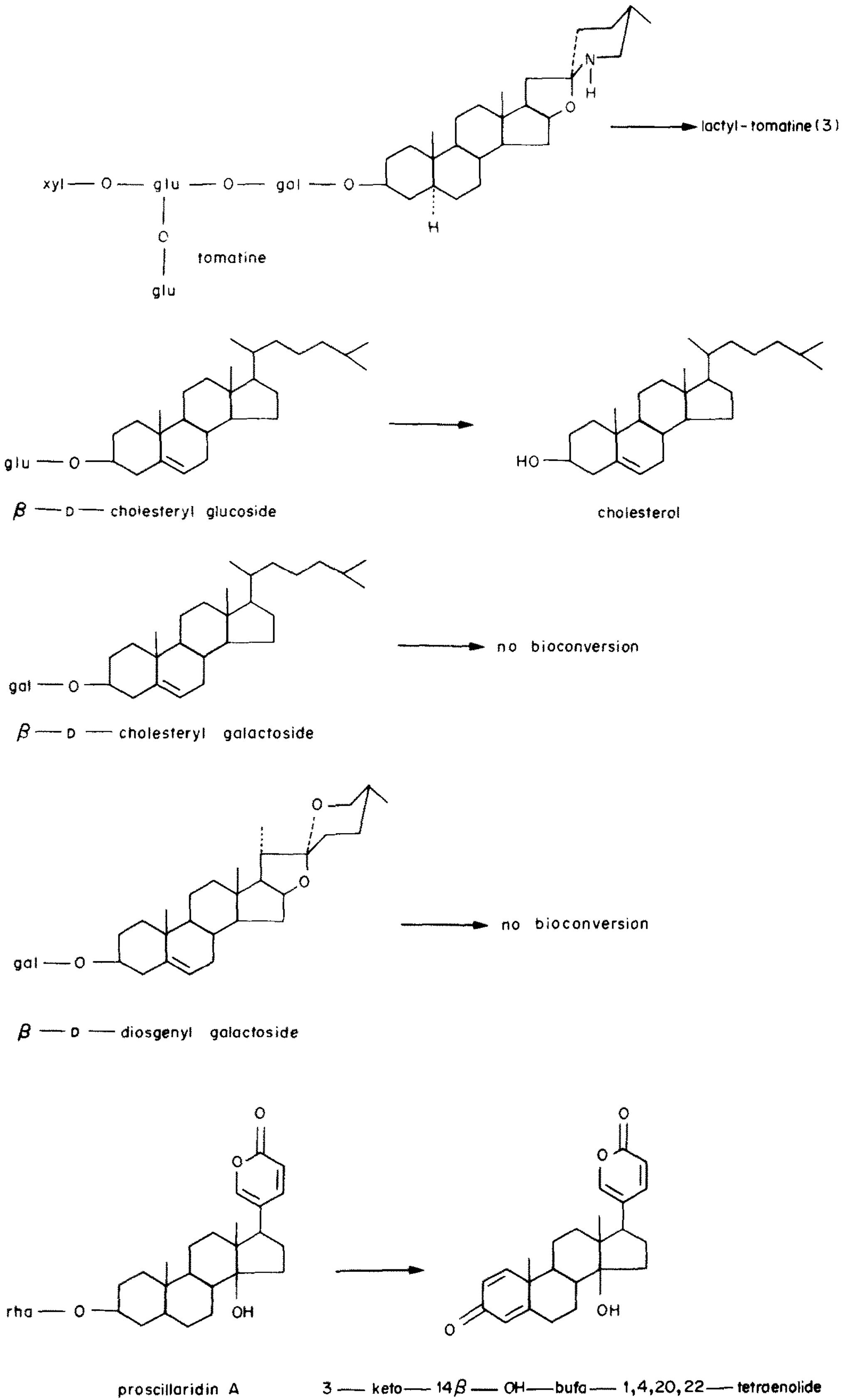

Fig. 1. Bioconversion of steroid glycosides by *Nocardia restricta*.

suspension in 0.4% NaCl solution. The incubation was continued for 72 h and the incubation broth was extracted with butanol. The analysis of the extract on silica gel GF_{254} in chloroform–methanol–1% NH_4OH (2:2:1, by vol.) solvent system showed no bioconversion products after spraying the plate with 50% H_2SO_4 in 90% ethanol.

RESULTS AND DISCUSSION

In order to determine the effect of the nitrogen atom on the bioconversion of steroidal glycosides, several steroid glycosides without the nitrogen atom in the molecule were incubated with *Nocardia restricta*. The substrates for bioconversion were the synthesized sterol glycosides β-D-cholesteryl glucoside and β-D-cholesteryl galactoside, the commercial heart glycoside from the group of bufadienolides proscillaridin A and the synthesized saponine β-D-diosgenyl galactoside.

The results of bioconversions, presented in Fig. 1, showed that *Nocardia restricta* converts steroidal glycosides differently according to the sugar bound to the steroid aglycone. β-D-cholesteryl galactoside and β-D-diosgenyl galactoside were not converted by *Nocardia restricta*. Steroid galactosides are not usually converted by microbes in contrast to glucoside which are, so these results would not at the first sight be unexpected. But since in tomatine the first sugar bound to the aglycone tomatidine is galactose and tomatine is readily conjugated with L(+)-lactic acid by *Nocardia restricta*, one would not expect the presence of galactose to be a barrier for bioconversion.

The other glycosides, β-D-cholesteryl glucoside and proscillaridin A, are bioconverted as normally expected. In both cases the glycosidic bond is cleaved and in the second case additional dehydrogenation in ring A is observed.

We can conclude, therefore, that for the conjugation of L(+)-lactic acid by *Nocardia restricta* with a steroid glycoside to occur the presence of a nitrogen atom in the molecule seems to be necessary.

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