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Note

Thin-layer chromatographic bioassay of iridoid and secoiridoid glucosides with a fungitoxic aglucone moiety using β-glucosidase and the fungus *Penicillium expansum* as a test organism*

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Subsequent use of the same silica gel thin-layer plate for chromatographic separation and for a biological assay using microbes as test organisms provides a very simple and rapid means of developing antimicrobial screening test for compound mixtures. Tests using different fungi and bacteria as test organisms to determine the activity of fungitoxic compounds (*e.g.*, phytoalexins) and antibacterial compounds, respectively, have been described[1–4].

Some compounds, however, are not active as such but only together with irradiation (photoactive compounds) or after enzymatic conversion. By carrying out a fungitoxicity bioassay on silica gel thin-layer plates and additionally subjecting the samples to UV irradiation at 366 nm, photoactive furocoumarins and furochromones can be determined very specifically and sensitively[5].

Many glycosides are known to be active as such but only together in the presence of their specific glycosidases, which split off the sugar moiety. This means that the glycosides as such may be considered as a prodrug form and that the corresponding aglycone or a related intermediate is responsible for the biological activity. Examples for this are the iridoid glucosides aucubin (I) and catalpol (II), which proved to be antimicrobial, but only in the presence of the enzyme β-glucosidase[6,7].

It has been shown previously that silica gel thin layers are very suitable for studying the hydrolysis of β-glucosides by β-glucosidase[8,9]. In this paper a method is reported that combines a chromatographic separation, an enzymatic conversion and an antimicrobial bioassay on the same silica gel thin-layer plate. The results obtained with some iridoid and secoiridoid glucosides including aucubin (I) and catalpol (II) using the enzyme β-glucosidase for their hydrolysis and the fungus *Penicillium expansum* as a test organism are presented.

* Part V in the series "Secoiridoids and xanthones in the genus *Centaurium*"; for Part IV see ref. 11. Part of these studies were presented at the *29th Annual Meeting of the Gesellschaft für Arzneipflanzenforschung, Marburg/Lahn, G.F.R., June 9–13th, 1981* [for summary, see *Planta Med.*, 42 (1981) 139].

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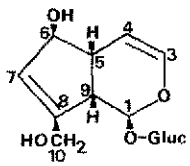
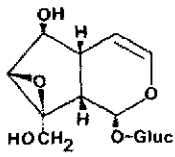
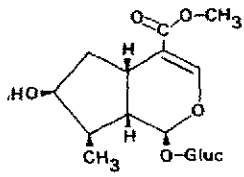
Iridoid glucosides



I Aucubin

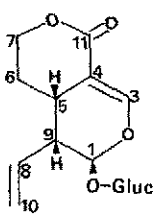


II Catalpol

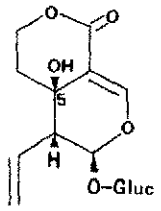


III Loganin

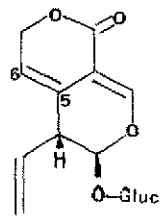
Secoiridoid glucosides



IV Sweroside



V Swertiamarin



VI Gentiopicrin
(=Gentiopicroside)

EXPERIMENTAL

Thin-layer chromatography

Pre-coated silica gel 60 F_{254} (10 × 10 cm) (Merck, Darmstadt, G.F.R.) and silanized silica gel F_{254} (10 × 10 cm) (Merck) TLC plates were used as sorbent layers.

The samples tested were the iridoid glucosides aucubin (Carl Roth, Karlsruhe, G.F.R.), catalpol (Carl Roth, Karlsruhe, G.F.R.) and loganin (donated by Professor Dr. H. Inouye, Kyoto, Japan) and the secoiridoid glucosides gentiopicrin (isolated from the roots of *Gentiana lutea* L.), swertiamarin (isolated from the aerial parts of *Centaurium littorale* Gilmour) and sweroside [isolated from the aerial parts of *Centaurium spicatum* (L.) Fritsch][10]. All glucosides were dissolved in methanol at concentrations of 2 mg/ml and 10 μl of each solution were applied as a spot.

The solvent system used was ethyl acetate–methanol–water (38.5:7.5:4). The thin-layer chromatograms were developed in a saturated chamber allowing the solvent to migrate a distance of 8 cm.

Detection was effected by using UV light at 254 nm, the spots being marked with a pencil. Next the plates were sprayed with a conidial suspension (see *Bioassay*) or with anisaldehyde–sulphuric acid reagent and heated at 105°C for 10 min.

Enzymatic hydrolysis on silica gel thin-layer plates

β-Glucosidase, Rotipuran® pulv ex amygdal (925 U/mg), was purchased from Carl Roth.

In order to hydrolyse the glucosides on thin-layer plates before chromatography, the part of the TLC plates where the samples had been applied was wetted by spraying it with a 1 mg/ml aqueous solution of enzyme. The plates were incubated in a humid atmosphere for 15 min and dried[9].

Bioassay

The fungus *Penicillium expansum* used as a test organism was maintained in culture on potato glucose agar[1]. After chromatography, the well dried TLC plates were sprayed with a suspension of conidial spores of the test organism in a nutrient medium[1,5], with or without adding β-glucosidase at a concentration of 1.0 mg/ml. The plates were incubated at 25°C in the dark in a humid atmosphere for 48 h. The results were evaluated by observing the presence or absence of inhibition zones (white spots on a green background) with light directed on to the plate at a very small angle from one side of two identically prepared chromatograms, one sprayed with a β-glucosidase solution and one without.

To determine the optimal enzyme concentration in the conidial spray suspension, concentrations of 2.0, 1.0, 0.5 and 0.25 mg/ml of β-glucosidase were tested.

To determine the minimal amount of a glucoside for which fungitoxicity is detectable under the described experimental conditions, volumes of 5 μl of sample dilution series containing 2.0, 1.0, 0.5, 0.25 and 0.1 mg/ml of glucoside in methanol were applied as spots on the TLC plates.

RESULTS AND DISCUSSION

As both the hydrolysis and the incubation of the conidial spores have to be carried out in a humid atmosphere, the simplest method for combining them is to add the β-glucosidase to the conidial suspension just before spraying. Tests with the glucosides carried out on silica gel thin-layer plates with different concentrations of β-glucosidase showed that the minimal concentration of the enzyme causing optimal inhibition zones is about 0.5–1.0 mg/ml. As a consequence, we used an enzyme concentration of 1.0 mg/ml of conidial spray suspension in the screening tests.

Fig. 1. shows the results obtained with the iridoid glucosides aucubin (I), catalpol (II) and loganin (III) and the secoiridoid glucosides sweroside (IV), swertiamarin (V) and gentiopicrin (gentiopicroside) (VI). None of these glucosides is fungitoxic towards *Penicillium expansum* without adding β-glucosidase (Fig. 1a). In the presence of β-glucosidase I, II and also VI show an inhibition zone (Fig. 1b). The inhibition zones caused by I and II, however, are diffuse, probably owing to the diffusion of the aglucone moiety in the humid atmosphere during incubation. III, IV and V were found not to be fungitoxic towards *Penicillium expansum* at the concentration tested, even in the presence of β-glucosidase.

The same tests were also carried out after the glucosides had been treated first on the thin-layer plate with the β-glucosidase for 15 min before chromatographing to check the hydrolysis of the glucosides under investigation (Fig. 2). Hydrolysis products could be detected from III, IV and VI, but not from I, II and V, the first two probably because of the high diffusion of their hydrolysis products on silica gel thin layers.

Investigation of the hydrolysis with β-glucosidase of the secoiridoid glucosides IV, V and VI on both silica gel thin layers and in aqueous solutions demonstrated a marked difference in susceptibility towards β-glucosidase, especially between VI and V. V is only partly and slowly hydrolysed, whereas VI is hydrolysed rapidly. IV is also hydrolysed but not as rapidly as VI. A quantitative evaluation will be published separately[11].

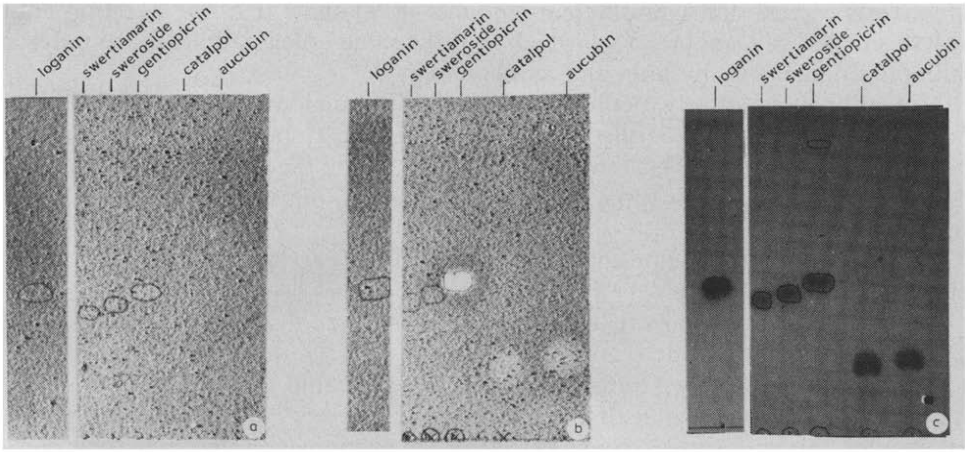


Fig. 1. Fungitoxicity bioassay of the iridoid glucosides aucubin (I), catalpol (II) and loganin (III) and the secoiridoid glucosides sweroside (IV), swertiamarin (V) and gentiopicrin (VI). Ringed areas show areas of compound spots detected under UV light (254 nm) before spraying with the conidial suspension. (a) Chromatogram sprayed with the conidial suspension; (b) chromatogram sprayed with the conidial suspension containing β-glucosidase; (c) chromatogram sprayed with anisaldehyde–sulphuric acid reagent.

Apart from glucose, only one hydrolysis product was detectable from both IV and III, which proved to be their respective aglucones (Fig. 2)[11]. From VI two hydrolysis products are detected (Fig. 2), of which the one with the highest R_F value proved to be gentiopicrin aglucone and the other one a conversion product of that aglucone[12]. The structural elucidation of this compound, an aldehyde, which we named gentiogenal, will be published elsewhere[12]. Gentiogenal is fungitoxic towards the test fungus (Fig. 2a and b).

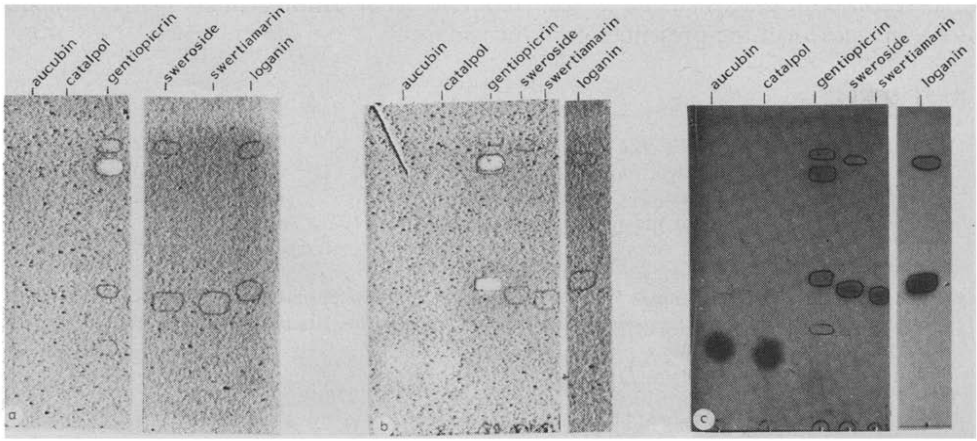


Fig. 2. Fungitoxicity bioassay of the iridoid glucosides aucubin (I), catalpol (II) and loganin (III) and the secoiridoid glucosides sweroside (IV), swertiamarin (V) and gentiopicrin (VI) and gentiopicrin (VI) after incubating the samples with β-glucosidase before chromatography. Ringed areas show areas of compound spots detected under UV light (254 nm) before spraying with the conidial suspension. (a) Chromatogram sprayed with the conidial suspension; (b) chromatogram sprayed with the conidial suspension containing β-glucosidases; (c) chromatogram sprayed with anisaldehyde–sulphuric acid reagent.

Tests carried out with different amounts of VI show that the detection limit with this bioassay is about 2.5 μg, which is of the same order as that for the polyene antibiotics natamycin, nystatin and amphotericin B[13].

Until now no other experimental data on the antimicrobial activity of VI were available other than its toxic effect on infusoria[6]. In this respect Hänsel[6] pointed out the structural similarities between the aglucone of VI and the antibiotic patulin[6]. The Merck Index[14] categorizes VI as an antimalarial agent, but without referring to experimental proof.

VI is one of the major constituents of *Gentianaceae* drugs, together with its related glucosides IV and V[15,16].

Among the chemically related secoiridoid and iridoid glucosides the described bioassay may be used to detect VI selectively on silica gel thin layers (Fig. 1b).

On silanized silica gel thin-layer plates the secoiridoid glucosides IV, V and VI can be separated much better than on silica gel thin layers[10]. On the former plates, however, no inhibition zones can be detected. The phenomenon that fungitoxic compounds, fail to show their activity in a bioassay on silanized silica gel has been described earlier[5,13].

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NOTE ADDED IN PROOF

Very recently gentiopicrin (VI) as well as I, II, III, IV, V and some other iridoid and secoiridoid glucosides have been reported to be antimicrobial against *Staphylococcus aureus* in the presence of β-glucosidase[17].

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