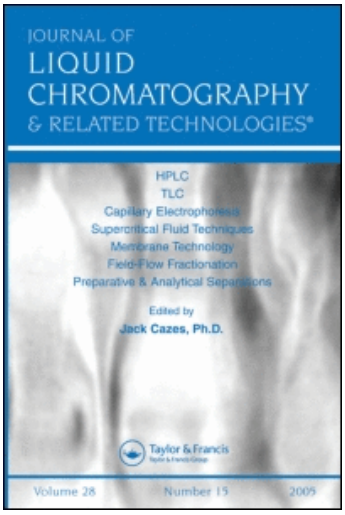

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 27 May 2004

To cite this Article Gosse, Benjamin K. , Ito, Yoichiro and Huang, Ru Chih(2004) 'Optimization of Active Saponin, Arganine C, for Microbicidal External Use', Journal of Liquid Chromatography & Related Technologies, 27: 12, 1947 — 1953
To link to this Article: DOI: 10.1081/JLC-120038780
URL: http://dx.doi.org/10.1081/JLC-120038780

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 12, pp. 1947–1953, 2004

Optimization of Active Saponin, Arganine C, for Microbicidal External Use

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ABSTRACT

The antiviral activities of the saponins, arganine C, and tieghemelin were described earlier [Gosse, B.K.; Gnabre, J.N.; Ito, Y.; Huang, R.C.; J. Liq. Chrom. Rel. Technol. **2002**, *25* (20), 3199–3211]. In this paper, conversion of tieghemelin to arganine C, a stronger antiviral entry saponin for a new microbicide is described. The crude saponin fraction (arganine C/ tieghemilin = 1 : 2) obtained by high-speed countercurrent chromatography

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DOI: 10.1081/JLC-120038780 1082-6076 (Print); 1520-572X (Online)

(HSCCC) [Ito, Y.: CRC Crit. Rev. Anal. Chem. **1986**, *17* (1), 65–143], was treated with 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in dimethylformamide (DMF) and refluxed for 5 hr. The reaction mixture shows a single peak which represents arganine C by HSCCC. From 700 mg of the crude sample, 500 mg of pure arganine was obtained.

Key Words: Tighemelin; Arganine C; Countercurrent chromatography; Saponin.

INTRODUCTION

Arganine C and tieghemelin (Fig. 1) are sugar-conjugated triterpenes isolated from the seeds of the ripe fruit of the rain forest tree, *T. heckelli*, and exhibit inhibitory activity against herpes viruses and HIV-1 entry at nontoxic concentrations.[1,3] At a higher dose, arganine C was found to be a better inhibitor of HSV-1 entry than tieghemelin. The antiviral activity of saponins has been well documented.[4] Several triterpenoid saponins from various plant species have been shown to be active against a number of viruses, including herpes viruses and HIV-1.[5]

Saponins have proven to be pharmacologically active agents and are major constituents of soybean food products.[6] The structure–activity relationship of saponins for the antiviral activity, however, is complex and appears to be specific for each saponin molecule. The present challenge is to produce arganine C in a large scale, as the principle active ingredient in the new microbicide formulation, where tieghemelin is used as templates to modify their chemical groups in such a way to eliminate the hemolytic side-effect without affecting their antiviral activity.

Since the sole difference between the two compounds is the sugar at the C-3 position, the glucuronic acid moiety on tieghemelin (a new feature in saponin chemistry) appears reducible into the alcohol group ($-CH_2OH$) (Fig. 1). This underscores the role of this chemical moiety as an important pharmacophore in HIV-1 attachment, since arganine C gives a more pronounced antiviral effect by the alcohol ($-CH_2OH$) function on the C-3 glucosyl residue.

EXPERIMENTAL

Apparatus

The cross-axis coil planet centrifuge[7] (a prototype fabricated at the National Institutes of Health, Bethesda, MD) was used for preliminary

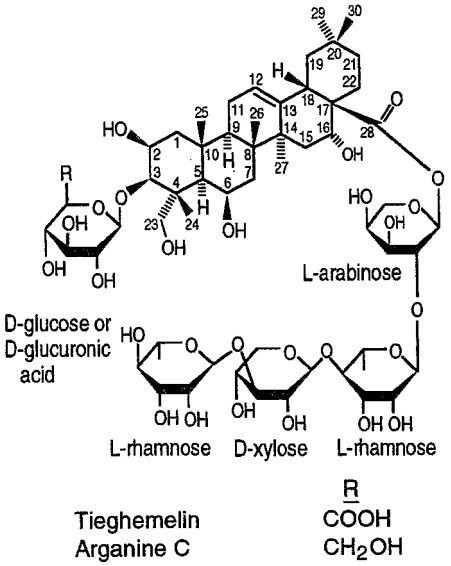

Figure 1. Chemical structures of tieghemelin and arganine C.

purification of crude extract. The apparatus holds a pair of multilayer coil separation columns at a distance 10 cm from the central axis of the centrifuge. In order to retain a satisfactory amount of the stationary phase, the column was mounted on the rotary shift 15 cm away from the mid point. Each column consisted of nine layers of left-handed coils of ca 50 m of 2.6 mm I.D. Teflon tubing. The beta value varies from 0.5 at the internal terminal to 0.75 to the external terminal. The two columns were connected in series to provide a total capacity of 570 mL. The revolution speed was adjusted at 650 rpm with a speed controller (Bodine Electric Company, Chicago, IL) for the present studies.

Reagents

Solvents used for high-speed countercurrent chromatography (HSCCC)[2] such as methyl *t*-butyl ether, 1-butanol, and acetonitrile were chromatographic grade and purchased from Fisher Scientific Co., Fair Lawn, NJ. Trifluoroacetic acid and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), $NaBH_4$ and dimethylformamide (DMF) were of reagent grade and obtained from Sigma Chemical Co., St. Louis, MO.

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HSCCC Separation

A two-phase solvent system composed of methyl *t*-butyl ether/1-butanol/acetonitrile/0.5%TFA aqueous solution (1 : 3 : 3 : 5, v/v/v/v) was prepared in a separatory funnel at room temperature, and the two phases separated shortly before use. The sample solution was prepared by dissolving 750 mg of the crude extract in 10 mL of the solvent consisting of about equal volumes of each phase.

The separation was performed as following: The column was first completely filled with the upper organic phase followed by sample loading from a pressured glass bottle. Then, the apparatus was rotated at 650 rpm, while the lower aqueous phase was eluted through the head end of the column at a flow rate of 3 mL/min. The effluent from the outlet of the column was continuously monitored through a UV detector (Uvicord S, LKB Instruments, Stockholm/Bromma, Sweden) at 280 nm, and collected into test tubes using a fraction collector (LKB Instruments).

RESULTS AND DISCUSSION

Reaction of Conversion

A crude saponin fraction from HSCCC (1.5 g), a semi-purified crude containing arganine C and tieghemelin at a ratio 1 : 2, was added to EEDQ (150 mg; 1.25 mmol) in DMF (5 mL), and the mixture was stirred at reflux for 5 hr. The solvent was removed using a rotary evaporator, and the brown residue was washed with diethyl ether (5 mL) to yield a brownish oily residue (160 mg) of ethyl ester intermediate. The residue was dissolved in ethanol (10 mL), and $NaBH_4$ (225 mg) was added, with stirring, at 0°C. After 16 hr at 25°C, methanol was added to decompose unreacted $NaBH_4$.

The mixture was filtered and the filtrate evaporated to give a brown solid, which was dissolved in 0.2 M ammonium acetate (50 mL) and extracted with butanol (2 × 50 mL). After drying the organic phase over Na_2SO_4, the solvent evaporation left 750 mg of white solid, in which the 1 : 2 ratio between the above two components was increased to >100 : 1 by TLC analysis. This reaction mixture was subjected to CCC purification as described below.

Purification of Arganine C from Tieghemelin

A sample (750 mg) of the reaction crude mixture was purified by solvent system HSCCC using a two-phase solvent system composed of methyl *t*-butyl ether (MtBE)/1-butanol/acetonitrile/0.5% TFA aqueous solution at a

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1 : 3 : 1 : 5 volume ratio. The multilayer coil separation column was eluted with the lower aqueous phase at a flow rate of 3 mL/min under 650 rpm.

Figure 2 shows the CCC separation of the crude extract containing arganine C and tieghemelin at a 1 : 2 ratio. The two saponin components are partially resolved and eluted in 2.5 hr. In Fig. 3 the HSCCC separation of the reaction mixture shows a single peak of arganine C. The major fractions containing the compounds of interest were pooled, and gave 500 mg of pure arganine C based on the TLC analysis.

CONCLUSION

The present study was conducted to increase the yield of the more active compound and avoid the need to separate arganine C from tieghemelin, the

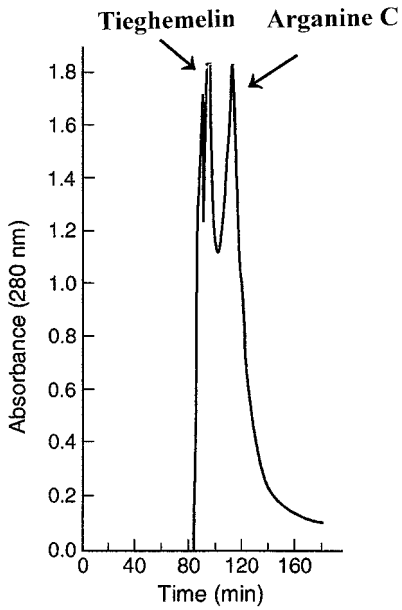

Figure 2. HSCCC separation of crude saponin sample. Experimental conditions are as follows: Apparatus: cross-axis coil planet centrifuge with 10 cm revolution radius; column: a pair of multilayer coils of PTFE (polytetrafluoroethylene) tubing, 2.6 mm I.D. with a total capacity of about 570 mL; sample: 750 mg of crude saponin mixture; solvent system: methyl *t*-butyl ether/1-butanol/acetonitrile/0.5% aqueous TFA (1 : 3 : 3 : 5, v/v/v/v); mobile phase: lower aqueous phase eluted head to tail; flow rate: 3 mL/min; detection: 280 nm.

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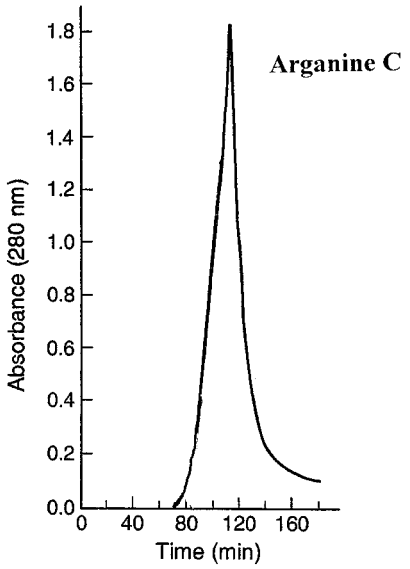

Figure 3. HSCCC separation of the reaction products of the crude saponin. Sample size: 750 mg. Other experimental conditions are described in Fig. 2 caption.

reduction of tieghemelin to arganine C was carried out on the saponin fraction of the extract. This was effected by making the ethyl ester with EEDQ,[8] and then reducing it with sodium borohydride.

Microbicides are substances which are useful for the public health for developing countries. But recent studies demonstrate that women showed a big need for alternative prevention methods. So, a recent inquiry of the Alan Guttmarcher Institute, estimates that 21 million of Americans are interested in a microbicidal product. In another study on acceptability led in Zimbabwe, Uganda, and South Africa, woman expressed willingness to possibly use a microbicide as demonstrated by Watts et al.[9]

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Received February 20, 2004
Accepted March 15, 2004
Manuscript 6331

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