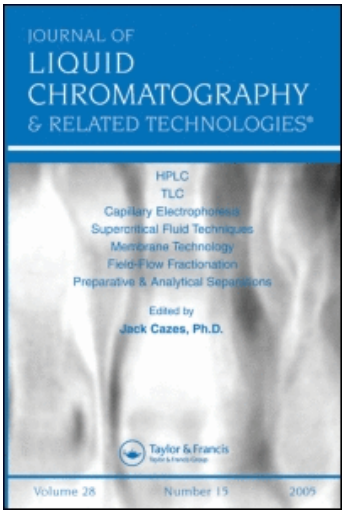

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

ISOLATION OF SAPONINS WITH VIRAL ENTRY INHIBITORY ACTIVITY BY COMBINED CHROMATOGRAPHIC METHODS

Benjamin K. Gosse[ab]; John N. Gnabre[a]; Yoichiro Ito[c]; Ru Chih Huang[a]
[a] Department of Biology, Johns Hopkins University, Baltimore, MD, U.S.A. [b] Department de Chimie, INP/HB, Yamoussokro, Cote d'Ivoire [c] Laboratory of Biophysical Chemistry, NHLBI National Institutes of Health, Bethesda, MD, U.S.A.

Online publication date: 26 November 2002

To cite this Article Gosse, Benjamin K. , Gnabre, John N. , Ito, Yoichiro and Huang, Ru Chih(2002) 'ISOLATION OF SAPONINS WITH VIRAL ENTRY INHIBITORY ACTIVITY BY COMBINED CHROMATOGRAPHIC METHODS', Journal of Liquid Chromatography & Related Technologies, 25: 20, 3199 — 3211
To link to this Article: DOI: 10.1081/JLC-120016662
URL: http://dx.doi.org/10.1081/JLC-120016662

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, No. 20, pp. 3199–3211, 2002

ISOLATION OF SAPONINS WITH VIRAL ENTRY INHIBITORY ACTIVITY BY COMBINED CHROMATOGRAPHIC METHODS

Benjamin K. Gosse,[1,2] John N. Gnabre,[1] Yoichiro Ito,[3] and Ru Chih Huang[1,*]

[1]Department of Biology, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218
[2]Department de Chimie, INP/HB, Yamoussoukro, BP 1083 Yamoussokro, Cote d'Ivoire
[3]Laboratory of Biophysical Chemistry, NHLBI National Institutes of Health, Bldg. 50, Rm 3334, 50 South Drive, Bethesda, MD 20892

ABSTRACT

High-speed countercurrent chromatography (HSCCC) is a support-free liquid–liquid partition chromatography (Ito, Y. Crit. Rev. Anal. Chem. 1986, *17* (1), 65–143). As an important separation technique, it eliminates complications between solutes and solid supports and has been used for preparative separation and purification for natural products in recent years. Using a series of chromatographic procedures, four triterpenoid saponins, arganine A, arganine C (Rev. 1), arganine D, and tieghemelin (Gen. 1) were isolated from the seed of the fruit of the rain forest tree,

*Corresponding author. Fax: (410) 516-5213; E-mail: huang@jhu.edu

DOI: 10.1081/JLC-120016662
1082-6076 (Print); 1520-572X (Online)

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

Tieghemella heckelli. In an initial pilot study, a HSCCC and preparative thin layer chromatography were used to isolate the herpesvirus entry inhibitors, Rev.1 and Gen.1, two compounds that are structurally identical except at position C-3 of the aglycone where in Rev.1 the sugar moiety is glucose and in Gen.1, it is glucuronic acid. Utilizing the charge difference between Rev.1 and Gen.1 at elevated pH, we were able to develop a simpler, more efficient purification scheme that eliminates the yield-limiting preparative TLC step used in the pilot study. Preparative amounts of purified Rev.1 (26 g or 0.78% yield) and Gen.1 (48 g or 1.43% yield) were isolated from 3.4 kg of *T. heckelli* seeds using cross-axis counter current chromatography (CCC) followed by partition chromatography in *n*-butanol : NH_4HCO_3, pH 7.9 and flash chromatography on florosil.

Key Words: Triterpenoid saponins; Arganine C; Tieghemelin; Counter current chromatography

INTRODUCTION

High speed countercurrent chromatography (HSCCC) has been increasingly used for the separation and purification of various natural and syntheic products and biological samples. The results of the present studies indicate that the HSCCC can be used for separation of saponins at both analytical and preparative scales. As saponins are highly streaky polar plant constituents, isolation is more difficult than for lower ones.[2] Various reversed-phase silica gels play an important role; and high performance liquid chromatography (HPLC) has been widely used, especially in the cases of minor saponins or saponins with delicate structures. This has promoted saponin study.

Tieghemella heckelii (Sapotaceae) or elephant tree grows in the rain forest of West Africa and various parts of the tree are widely used in traditional medicine in the Ivory Coast. The dry seed of the ripe fruit is a source of terpenes, fatty acids, and oil that is used for consumption and also serves as an effective treatment for children suffering from nutritional deficiency. Extracts of the bark of the tree and the roots are used as treatments for malaria, stomach ulcers, and skins disorders. Anecdotal accounts of anti-herpetic properties associated with the seed extract prompted a search for the individual molecular components responsible for this activity.

We have used a high-throughput herpesvirus entry assay[3] as a reference screening method and isolated a family of triterpenoid saponins, arganine A, arganine C (Rev. 1), arganine D, and tieghemelin (Gen. 1) from the defatted

extract of the seeds of *T. heckelli.* Arganine A and arganine D have previously been described[4] but were not assigned to a specific activity. Arganins C was first isolated from the fruit of *Argania spinosa* (Sapotaceae), a tree endemic to Morocco and also from the roots of the South African medicinal shrub, *Crossopteryx febrifuga* (Rubiaceae).[5] Tieghemelin (Gen. 1) is here described for the first time as a new saponin. Rev. 1 and Gen. 1 were found to be strong inhibitors of viral entry and their effects on HSV and HIV have been reported elsewhere.[6] Since the chemical synthesis of these structurally complex compounds is difficult and because we anticipate the need to produce them in large amounts eventually for clinical testing, we developed a large scale isolation procedure which involves a combination of chromatography methods. The key step in this procedure is the partitioning of Rev. 1 and Gen. 1 in *n*-butanol : NH_4HCO_3, made possible by a difference in charge between these two molecules at mild basic pH.

EXPERIMENTAL

Plant Material

The ripe fruit of *T. heckelii* was collected in the month of November in the Ivory Coast. The fresh pulp (mesocarp) of the fruit was removed and the hard shell (endocarp) was opened to collect the seeds which were sun-dried and then ground in a screen Wiley mill. In the pilot study, 250 g of the seed powder was used. For the preparative purification scheme, 3.360 g were processed.

Apparatus

The HSCCC centrifuge with Ito multiplayer coil has been previously described.[7] The versatile cross-axis coil planet centrifuge (CPC) was initially described by Shinomiya et al.[8] The solvent was pumped with a Milton Roy metering pump (Model 196-31; LTC/Milton Roy, FL). The eluent was monitored at 254 nm with a UV detector (Model Uvicord S, LKB Instrument, Stockholm, Sweden) with a strip chart recorder (LKB Instruments).

All fractions were analyzed by silica gel TLC plates (BAKER Si500F) using the solvent system, chloroform : methanol : 0.5%TFA (60 : 40 : 5). The TLC plates were visualized by cerium sulfate charring [2% $CeSO_4$ (w/v) in 5.6% H_2SO_4 (v/v)].

The structure of purified compounds was elucidated by NMR and mass spectrometry (MS).[9]

High Speed Countercurrent Chromatography Separation

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 two phases separated shortly before use. The sample solution was prepared by dissolving 18 g of the crude extract in 100 mL of the solvent consisting of about equal volumes of each phase.

The separation was performed as follows: 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 of the column at a flow rate of 3 mL/min. The effluent from the potlet 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).

Herpesvirus Type 1 Entry and Infectivity Assay

The Herpesvirus type 1 (HSV-1) entry and infectivity assay used to monitor the fractionation was originally established by Montgomery et al.[3] and makes use of stably transfected CHO cells (CHO-HveA) constitutively expressing the HSV entry mediators HveA and harboring the β-galactosidase reporter gene linked to the HSV ICP4 promoter for detection of HSV-1 entry. Fractions to be tested were dried, resuspended in PBS and added to the CHO-HveA growth media prior to challenging the cells with HSV-1.

RESULTS

General Isolation

The procedure began with a three cycle-maceration of 3.360 g of the seed paste with hexane. As shown in Fig. 1A, this defattation removed nearly 1.26 kg (38%) of hexane-soluble substances. Based on the water-solubility of the two glycosides, the marc of the seeds was first treated with water before methanol extraction. This step improved the yield of Gen.1 which is more water soluble than Rev.1. Methanol extraction of the dry aqueous residue yielded 270 g of the parent crude extract referred to as CCo of which 18 g was further purified with the cross-axis countercurrent chromatography (CCC) using the two-phase solvent system described earlier. However, this time, the aqueous layer was chosen as a mobile phase inverting the elution peaks (the more hydrophilic

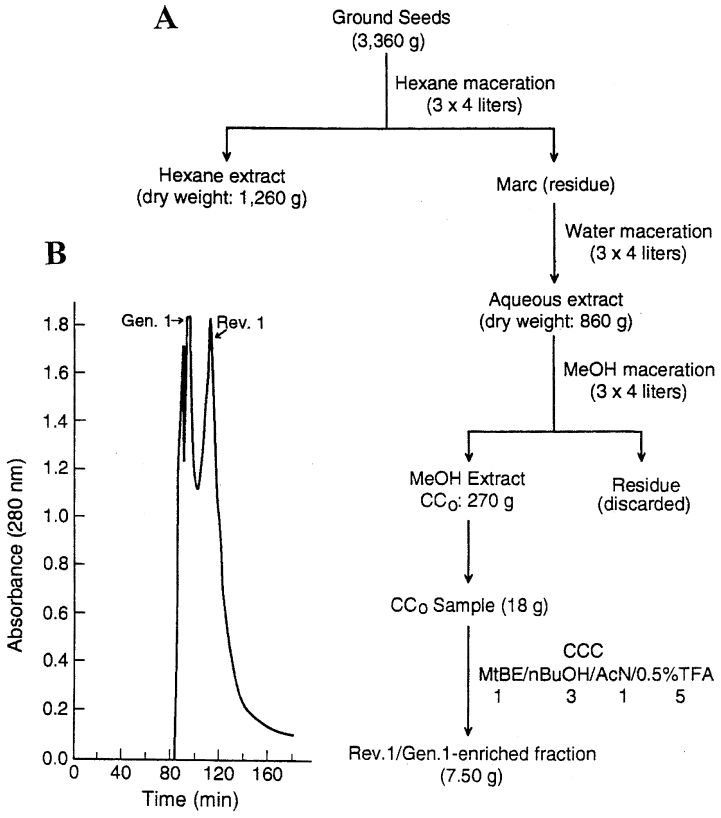

Figure 1. (A) Large scale isolation of a Rev.1/Gen.1-enriched fraction from the seeds of *T. heckelii.* The ground seed paste (3.36 kg) was macerated three times with hexane, removing 1.26 kg of hexane-soluble materials. The marc was extracted with water and the dry aqueous extract was treated with methanol to obtain a MeOH-soluble residue referred to as CCo (860 g). A 18-g sample of CCo was purified by Cross-axis CCC using the solvent system, methyl *ter*-butyl ether (MtBE) : butanol (nBuOH) : acetonitrile (AcN) : 0.5%TFA in 1 : 3 : 1 : 5 ratio (B) Two peaks enriched in Gen. 1 and Rev. 1 were resolved by this method. These were pooled, yielding a 7.50 g fraction.

compound, Gen. 1, appearing first) as shown in Fig. 1B. The TLC analysis of CCC fractions generated by the 18 g fractionation of CCo (Fig. 1A) revealed that the two peaks (Gen. 1 and Rev. 1) were still a mixture: the first peak was mainly Gen. 1 but retained 20–30% Rev. 1, and vice-versa. Therefore, the two peaks were pooled and dried, generating 7.50 g of a Rev. 1/Gen. 1-enriched residue.

Fractionation of CCo and Isolation Rev. 1 and Gen. 1 CCC and Preparative TLC

A working CCo fraction was generated by hexane deffatation the seeds from *T. heckellii*, (Fig. 1A) and tested for HSV-1 entry inhibition. At a single concentration (30 μg/mL), the CCo fraction showed 81% activity whereas the hexane-soluble fraction was devoid of activity. A pilot study on 100 mg of CCo was undertaken using the HSCCC with the Ito coil.[8] The reference solvent system that gave an adequate partition coefficient was the two-phase system, methyl *ter*-butyl ether (MtBE) : butanol (*n*BuOH) : acetonitrile (ACN) : 0.5%TFA in 1 : 3 : 1 : 5 ratio. This operation (Fig. 2) generated several fractions (F1–F6) which were pooled according to their TLC patterns and their activity in the bioassay. Fraction F5 which showed a high antiviral entry activity, was homogenous with an $R_f = 0.3$ in the TLC solvent system, chloroform : methanol : 0.5%TFA (60 : 40 : 5). F5 was therefore submitted to structure analysis by NMR and GC-mass spectrometry. These studies proved F5 to be Arganine C, referred herein as Rev.1 (for *Revelation 1*) and found it to be >99% pure.

In contrast, two major components with $R_f = 0.08$ and $R_f = 0.3$, present in the relatively large fraction F6 (28.7 mg), could not be resolved by CCC despite several attempts of optimization of the reference solvent system. The two components were instead resolved by preparative TLC with MeOH extraction. The NMR studies confirmed the $R_f = 0.3$ component to be Rev. 1 which was only partially recovered in fraction F5. The other more polar ($R_f = 0.08$) component was found to be a new triterpenoid saponin denoted, tieghemelin, and herein termed Gen. 1 (for *Genesis 1*). This compound was much less active in HSV-1 entry assay. Overall, the preparative TLC method afforded 16.5% of Rev.1 and 12.5% of Gen. 1 from the initial 100 mg CCo. The detailed structure analysis of these compounds have been described[9] along with their antiviral activities.[6] With this method, the two saponins occur in the seed of the ripe fruit with a % yield approximately of 0.38% (for Rev. 1) and 0.29% (for Gen. 1) based on the initial 250 g of the seed weight.

Isolation of Other Saponins from the CCo Fraction

A 3-g sample of CCo (a relatively complex mixture) was partitioned in butanol : 0.2 M ammonium acetate (NH_4OAc); pH = 6.7. After 10 min of vigorous shaking and centrifugation, the organic layer was carefully separated from the aqueous phase. A 500-mg (dry weight) residue of the organic phase was further purified by CCC using the same two-phase system, MtBE : *n*BuOH : AcN : 0.5%TFA (1 : 3 : 1 : 5) but in the presence of 2% NaCl. Here, the organic layer was chosen as a mobile phase while the aqueous layer served as a stationary

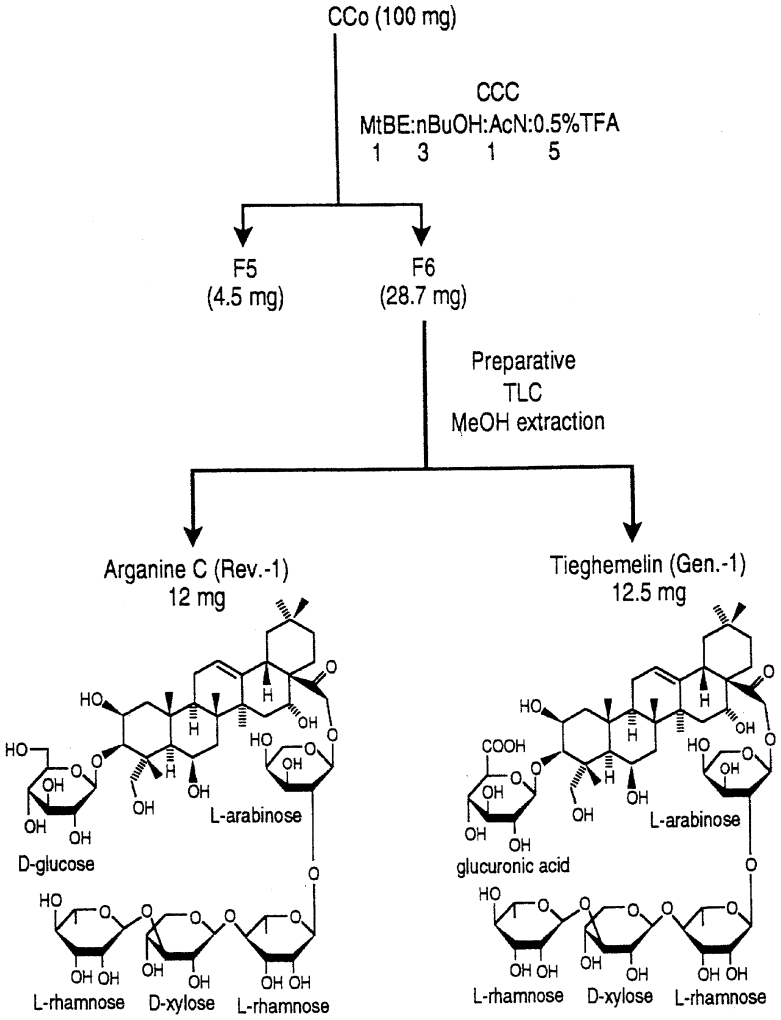

Figure 2. Pilot study for the isolation of viral entry inhibitors from the seeds of *T. heckelii.* Hundred milligram sample of CCo purified by HSCCC with the analytical Ito coil, generated several fractions (F1 to F6) including a reasonably pure and active F5 component of $R_f = 0.3$ found by NMR analysis to be arganine C (Rev. 1). In contrast, fraction F6 was a mixture of two nearly homogenous components of $R_f = 0.08$ and $R_f = 0.3$. This mixture was resolved by preparative TLC with MeOH extraction. NMR analysis proved the $R_f = 0.08$ component to be a new triterpenoid saponin termed tieghemelin (Gen. 1). The same analysis confirmed the second ($R_f = 0.3$) component to be arganine C. An overall yield of 0.38% for Rev. 1 and 0.29% for Gen. 1 was achieved from this pilot study.

phase. This operation generated several fractions which by TLC analysis consisted of three relatively homogenous components (Fa, Fb, and Fc). The NMR studies revealed Fa to be arganine A, Fb to be arganine C (Rev.1), and Fc to be arganine D. These compounds occur in fairly high yield as shown in Fig. 3.

Effects of the Products on Herpesvirus Type 1 Entry and Infectivity

HveA is one of the major cell surface receptors for HSV-1 entry. The purified saponins along with the parent CCo fraction were assayed for anti-HSV-1 entry activity mediated by HveA. Figure 4 illustrates the results of these studies. The concentration at which Rev.1 exhibited 50% inhibitory activity (IC_{50}) was 8.5 μg/mL. In contrast, CCo fraction had an IC_{50} of 20 μg/mL. This reflects nearly a 2.5-fold enrichment of the most active principle. The inhibitory activity of Gen.1 barely reached 50% at 40 μg/mL, a concentration at which cell viability was significantly decreased. Arganine A and arganine D were devoid of activity (data not shown). It should be noted that in other studies,[7] Gen. 1 and Rev. 1 had similar inhibitory activities against HIV-1 entry and infectivity at a concentration range non toxic to cells.

Optimization of Active Saponins Isolation

A small sample (150 mg) of this residue was partitioned in *n*BuOH : NH_4HCO_3 ($pH = 7.9$) since ammonium bicarbonate at $pH = 7.9$ proved to be more efficient at ionizing the carboxylic moiety of Gen. 1 than the ammonium acetate ($pH = 6.7$) used initially (Fig. 5). The two layers resulting from this partition were separated and dried. The Rev.1-enriched organic phase (54 mg) and the aqueous layer in Gen. 1 (95 mg) were further purified by flash chromatography (Fig. 4C) using florisil as a matrix and chloroform : MeOH : 0.5%TFA (6 : 4 : 0.5) for elution. This fractionation scheme, remarkable by its simplicity, afforded 35 mg (0.78%) of Rev. 1 and 64 mg (1.43%) of Gen. 1, and was selected for a scale up purification of the rest of the CCC-generated fraction enriched in Rev. 1 and Gen. 1.

DISCUSSION

A current trend in antiviral research focuses on development of viral entry inhibitors targeted at cell surface receptors. These receptors being of host origin, are stable and immutable, and therefore, are prone to virtually little or no viral resistance. In the present studies, we have demonstrated that the seed of the ripe

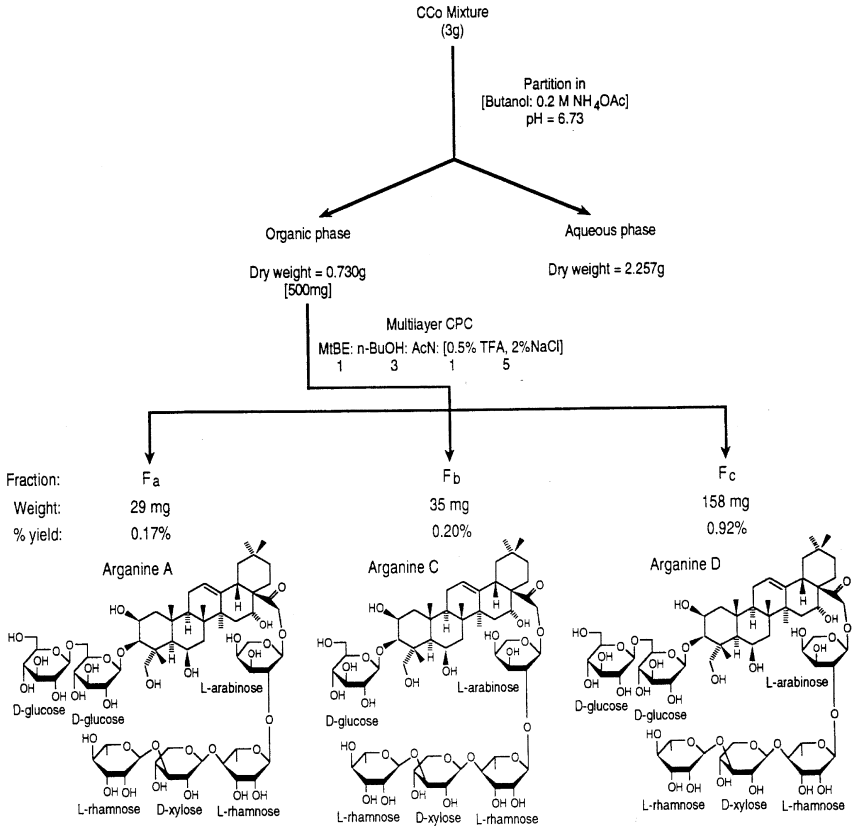

Figure 3. Inhibition of HSV-1 entry by Rev. 1 and Gen. 1. For inhibition of HSV-1 entry, CHO cells expressing the entry mediator HveA and an ICP4 promoter-driven β-galactosidase reporter gene were incubated with graded drug concentrations of Rev. 1, Gen. 1, and CCo for 30 min after which time, cells were challenged with HSV-1 (KOS) at 100,000 pfu/well. The reaction mixtures were incubated for an additional 7 hours to allow for VP16 transactivation and β-galactosidase expression to occur. Cells were then lysed by adding 50 µL of 1% nonidet P-40 in PBS with 3 mg/mL of *o*-nitrophenyl-β-D-galactopyranoside substrate. After a 15-min incubation at room temperature, the β-galactosidase activity was measured at 570 nm on a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments). The background OD values from the negative control CHO-IEβ8 cells were subtracted from test sample values and the % inhibition was calculated as follows: $100 - [(\text{Drug-treated Sample})/(\text{Untreated Sample}) \times 100]$. Each point represents the average of eight determinations for two independent experiments run in quadruplicate.

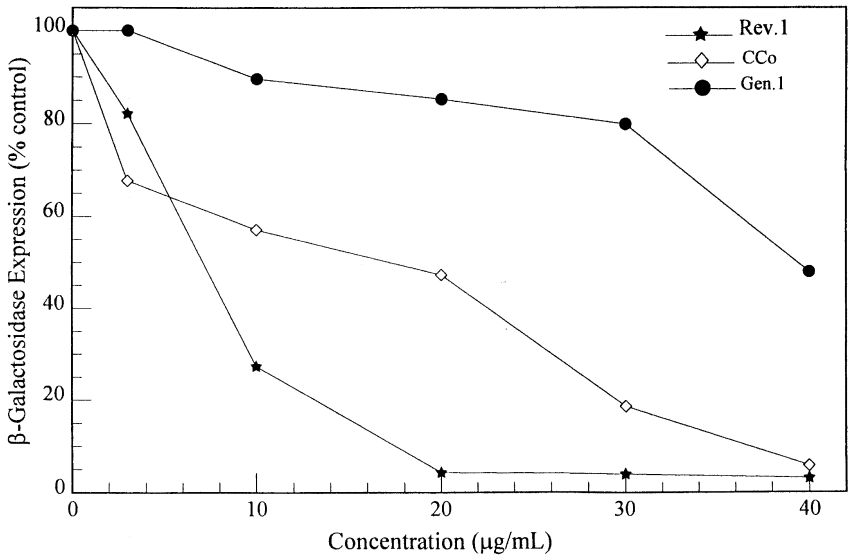

Figure 4. Identification of arganine A, C, D by partition chromatography of CCo at pH 6.7. A 3-g sample of CCo was partitioned in butanol : 0.2 M NH_4OAc, pH = 6.7. After 10 min shaking and centrifugation of the mixture, the organic layer was separated from the aqueous phase. The two layers were dried, affording 0.730 g of organic residue and 2.257 g of an aqueous brown substance. A 500-mg sample of the organic residue enriched in arganine A, C, D and deficient in tieghemelin, was further purified by CCC using MtBE : *n*BuOH : AcN : 0.5%TFA, 2%NaCl in 1 : 3 : 1 : 5 ratio. Three major fractions (Fa, Fb, and Fc) were pooled, based on their TLC patterns. NMR analysis revealed Fa to be arganine A, Fb to be arganine C (Rev. 1), and Fc to be arganine D.

fruit of the rain forest tree, *T. heckellii*, contain triterpenoid saponins with inhibitory activity towards HSV-1 entry. These saponins were initially resolved by a series of chromatographic methods. Optimization of the isolation conditions led to the elimination of the cumbersome preparative TLC step, affording a two-fold increase in the yield of active compound (Rev. 1) and 5-fold the yield of tieghemelin (Gen. 1). Genesis 1 is a new saponin that has exhibited reduced toxicity throughout our antiviral studies.[10] Furthermore, this compound has been shown to be as active as Rev. 1 against HIV-1 entry and infectivity.[6] These two molecules may therefore represent a chemotype for the development of a new class of inhibitors of viral entry. Finally, we observed that the seed of the ripe fruit used in the current studies contains more fatty acids and oily substances (38%), and less saponins than has been found for the green fruit. Saponins have proved to be pharmacologically active agents, however, the high hemolytic index and the

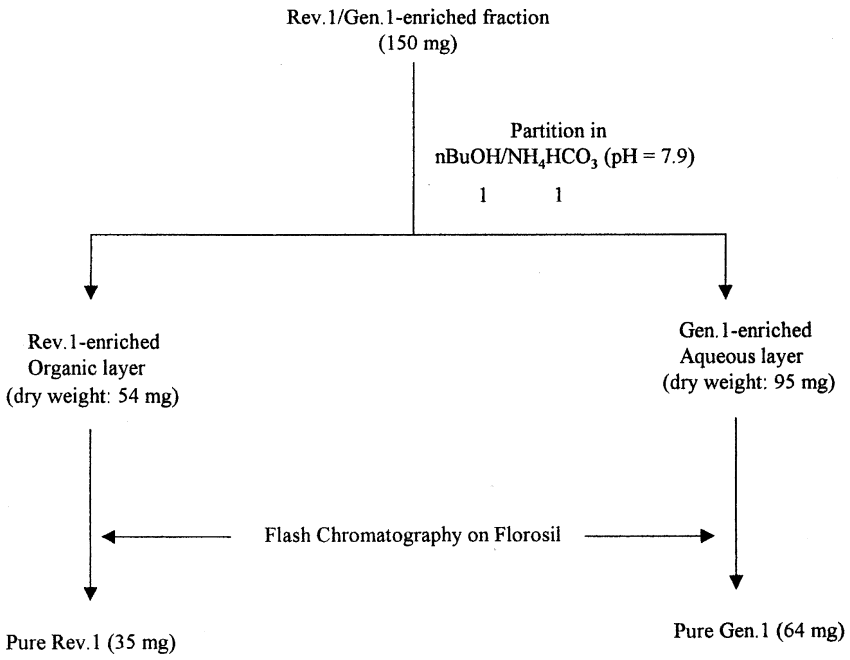

Figure 5. Isolation of Rev. 1 and Gen. 1 by liquid partition and flash chromatography. Rev. 1/Gen. 1-enriched fraction, A 150-mg (dry weight) residue of the Rev. 1/Gen. 1-enriched fraction was partitioned in *n*BuOH : NH_4HCO_3, pH = 7.9. The two non-miscible layers were separated and dried. The Rev. 1-enriched organic phase (54 mg) and Gen. 1-enriched aqueous layer (95 mg) were separately purified by flash chromatography on florisil matrix using chloroform : MeOH : 0.5%TFA (6 : 4 : 0.5) as an eluant. This operation afforded 35 mg of Rev. 1 and 64 mg of Gen. 1 from 150 mg sample, which represents an overall yield of 0.78% and 1.43%, respectively, based on the original weight of the plant seeds.

cytotoxicity associated with some classes of saponins has restricted their widespread clinical use.[11] Not all saponins are hemolytic,[12] therefore using the optimized isolation conditions described here, we are investigating the saponins present in the young fruit in attempts to discover new molecules with strong anti-viral entry activity and less cytotoxic effects.

CONCLUSION

The optimized isolation conditions are coupled with the high capacity Cross-axis CPC, the overall yield based on the 3.36 kg of plant seeds is 26 g

(0.78% yield) for Rev.1 and 48 g (1.43% yield) for Gen. 1. Compared to the preparative TLC method, these yields represent a 2-fold and a 5-fold increase for Rev. 1 and Gen. 1, respectively. The complex chemical structures of saponins present a considerable challenge for organic synthesis. The remarkably high yield generated from our optimized method, should enable further antiviral studies with Rev. 1 and Gen. 1 both in cell cultures and animal models without the need for chemical synthesis. When the optimized isolation conditions are coupled with the high capacity Cross-axis CPC, the overall yield based on the 3.36 kg of plant seeds is 26 g (0.78% yield) for Rev. 1 and 48 g (1.43% yield) for Gen. 1. Compared to the preparative TLC method, these yields represent a 2-fold and a 5-fold increase for Rev. 1 and Gen. 1, respectively. The complex chemical structures of saponins present a considerable challenge for organic synthesis. The remarkably high yield generated from our optimized method, should enable further antiviral studies with Rev. 1 and Gen. 1 both in cell cultures and animal models without the need for chemical synthesis.

ACKNOWLEDGMENTS

This work was supported by a grant to R.C. Huang from NIH (1RO1DE12165) and Biocure Medical LLC. We would like to thank Dr. David Mold for his comments on this manuscript.

REFERENCES

1. Ito, Y. Crit. Rev. Anal. Chem. **1986**, *17* (1), 65–143.
2. Yang, Chong-Reng; Li, Xing-Cong. *Saponins Used in Traditional and Modern Medicine*; Waller, Yamasaki, Eds.; Plenum Press: New York, 1996; 225–238.
3. Montgomery, R.I.; Warmer, M.S.; Lum, B.J.; Spear, P.G. Cell **1996**, *87*, 427.
4. Gariboldi, P.; Verotta, L.; Gabetta, B. Phytochem. **1990**, *29*, 2629.
5. Charrouf, Z.; Wieruszeski, J.M.; Fkih-Tetouani, S.; Leroy, Y.; Charrouf, M.; Fournet, B. Phytochem. **1992**, *31*, 2079.
6. Gnabre, J.N.; Gosse, B.K.; Park, R.; Gallay, P.; Huang, R.C. Antivir. Res. submitted for publication.
7. Mandava, N.B.; Ito, Y. In *Countercurrent Chromatography: Theory and Practice*; Mandava, N.B., Ito, Y., Eds.; Marcel Dekker: New York, 1988; App. 4, 823 pp.
8. Shinomiya, K.; Menet, J.P.; Fales, H.M.; Ito, Y. J. Chromator. **1993**, *644*, 215.
9. Gosse, B.K.; Gnabre, J.N.; Bates, R.B.; Dicus, C.W.; Nakkiew, P.R.C. J. Nat. Prod. **2002**.

10. Warner, M.S.; Geraghty, R.J.; Martinez, W.M.; Montgomery, R.I.; Whitbeck, J.C.; Xu, R.; Eisenberg, R.J.; Cohen, G.H.; Spear, P.G. Virol. **1998**, *246*, 179.
11. Hostettmann, K.; Marston, K.A. Ed. In *Chemistry and Pharmacology of Natural Products—Saponins*; Cambridge University Press: New York, 1995; 232.
12. Yoshiki, Y.; Kudou, S.; Okubo, K. Biosci. Biotechnol. Biochem. **1998**, *62*, 2291.

Received September 3, 2002
Accepted September 28, 2002
Manuscript 5952