

## Supporting Information

### Drug Leads from Endophytic Fungi: Lessons Learned via Scaled Production

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**Fig. 1S.** Polyhydroxyanthraquinones previously isolated from *Penicillium restrictum* (strain G85).

**Fig. 2S.** *Penicillium restrictum* (strain G85) grown on different media and pH conditions.

**Fig. 3S.** *Penicillium restrictum* (strain G85) on commercial rice (variety: Calrose Botan) using Sabouraud dextrose broth and YESD broth.

**Fig. 4S.** Quantity of  $\omega$ -hydroxyemodin (**1**) obtained after 4 wk growth on various media.

**Fig. 5S.** Recovery of  $\omega$ -hydroxyemodin (**1**) from the aqueous phase of a CHCl<sub>3</sub> partition.

**Fig. 6S.** Stages in generating the initial extract.

**Fig. 7S.** Evaluation of the purity of a batch of  $\omega$ -hydroxyemodin (**1**) obtained using the methods discussed in this paper.

**Fig. 8S.** <sup>1</sup>H NMR spectrum of  $\omega$ -hydroxyemodin (**1**) evaluated in DMSO-*d*<sub>6</sub> on a 500 MHz instrument.

**Fig. 9S.** Single injection per point calibration curve for  $\omega$ -hydroxyemodin (**1**) in  $\mu\text{g}/\text{mg}$  of sample.

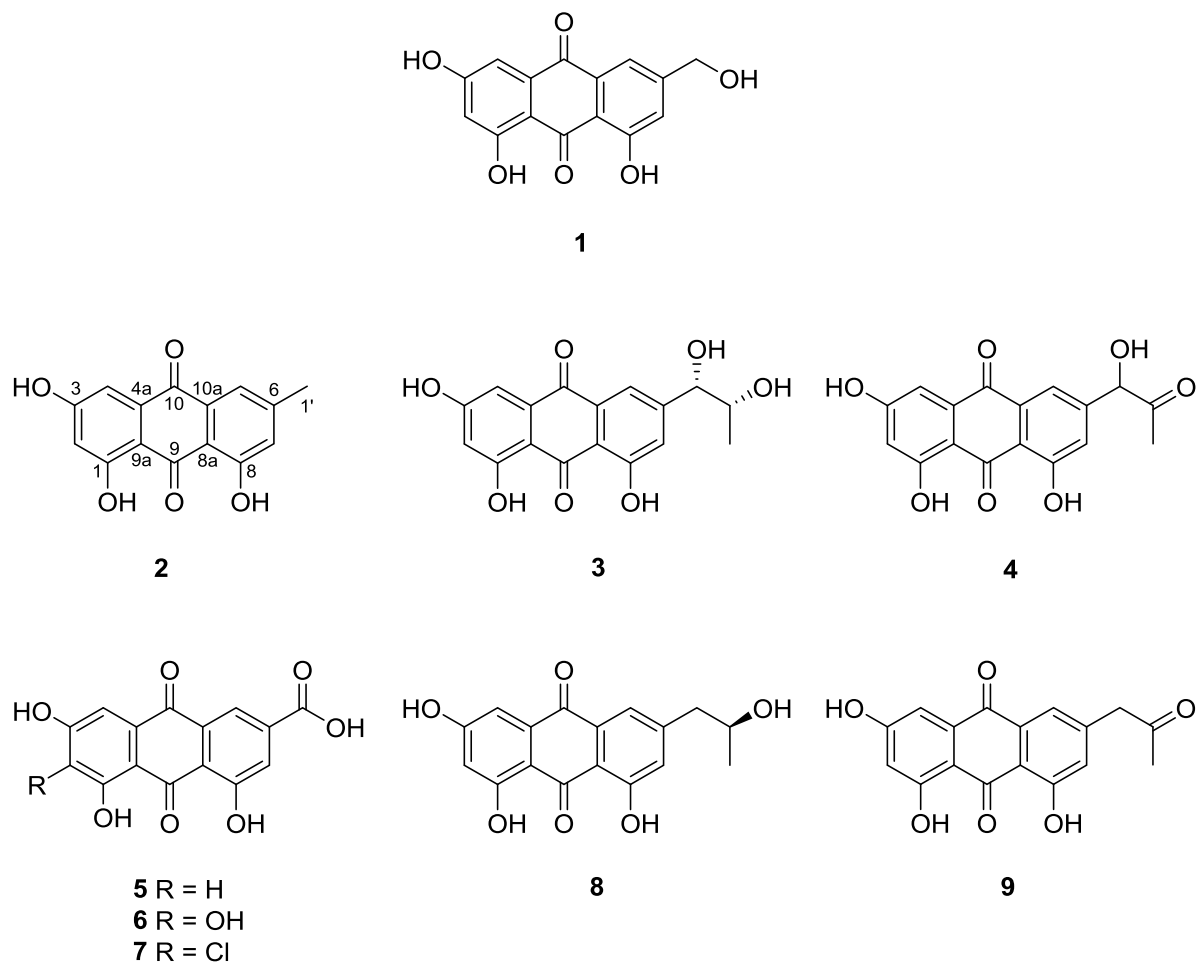
**Fig. 10S.** Initial fractionation of G85 extract depending on  $\omega$ -hydroxyemodin (**1**) content.

**Fig. 11S.** Improvements in the extraction step of the purification of  $\omega$ -hydroxyemodin (**1**).

**Fig. 12S.** Improvements in the partitioning steps of the purification of  $\omega$ -hydroxyemodin (**1**), serving to increase the recovery and purity of **1** through the process.

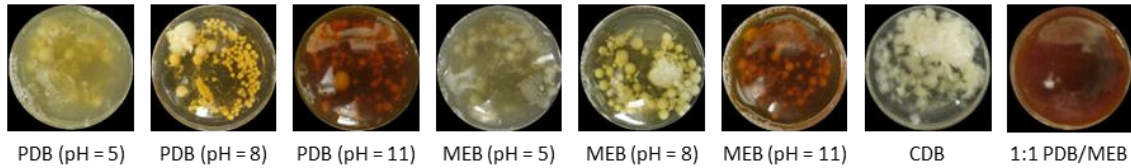
**Protocol 1S.** Example extraction procedure for the isolation of  $\omega$ -hydroxyemodin (**1**) rich extracts.

**Fig. 1S.** Polyhydroxyanthraquinones previously isolated from *Penicillium restrictum* (strain G85), including  $\omega$ -hydroxyemodin (**1**), emodin (**2**), 1'-hydroxyisorhodoptilometrin (**3**), 1'-hydroxy-2'-ketoisorhodoptilometrin (**4**), emodic acid (**5**), 2-hydroxyemodic acid (**6**), 2-chloroemodic acid (**7**), (+)-2'S-isorhodoptilometrin (**8**), and desmethyl dermoquinone (**9**).

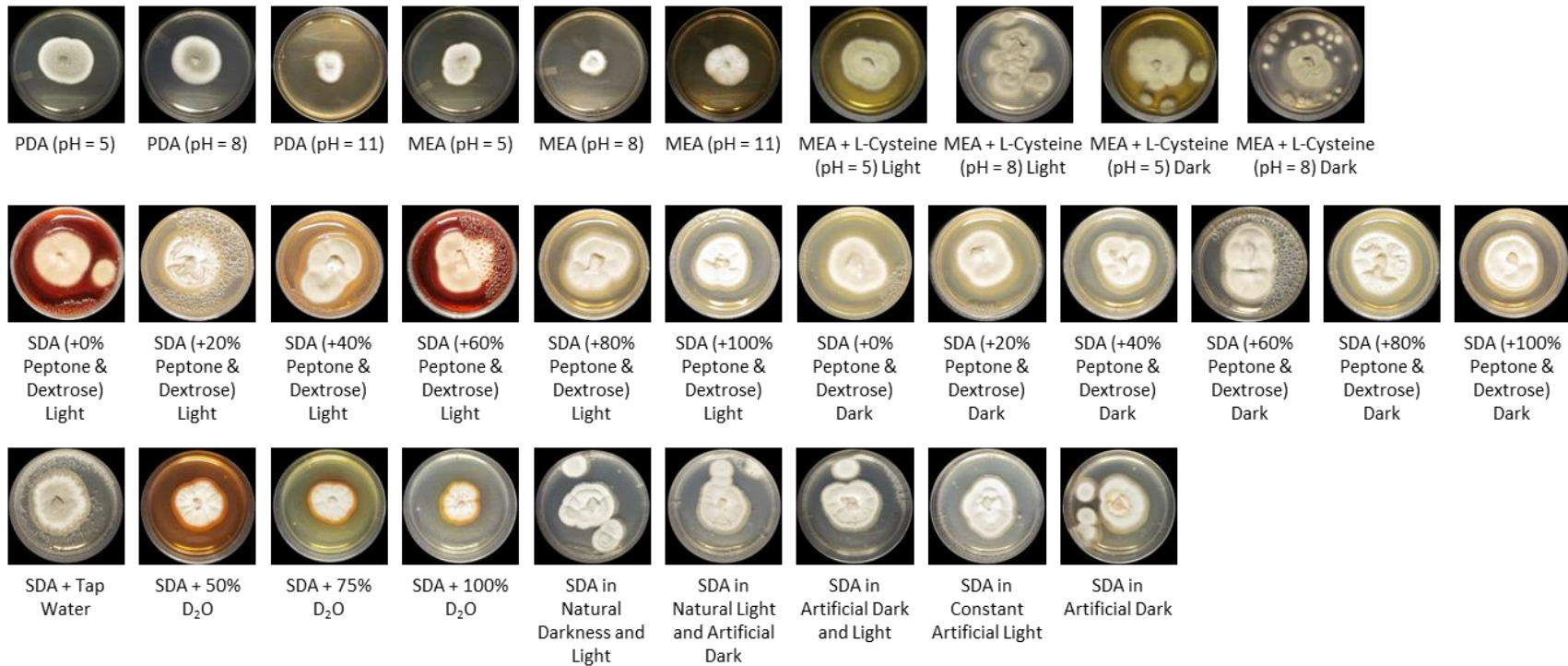


**Fig. 2S.** *Penicillium restrictum* (strain G85) grown on different media and pH conditions. PDB: potato dextrose broth; PDA: potato dextrose agar; MEB: malt extract broth; MEA: malt extract agar; CDB: Czapek Dox broth; SDA: Sabouraud dextrose Agar; Light: light/dark cycles; Dark: constant darkness

**Liquid/Broth Cultures**



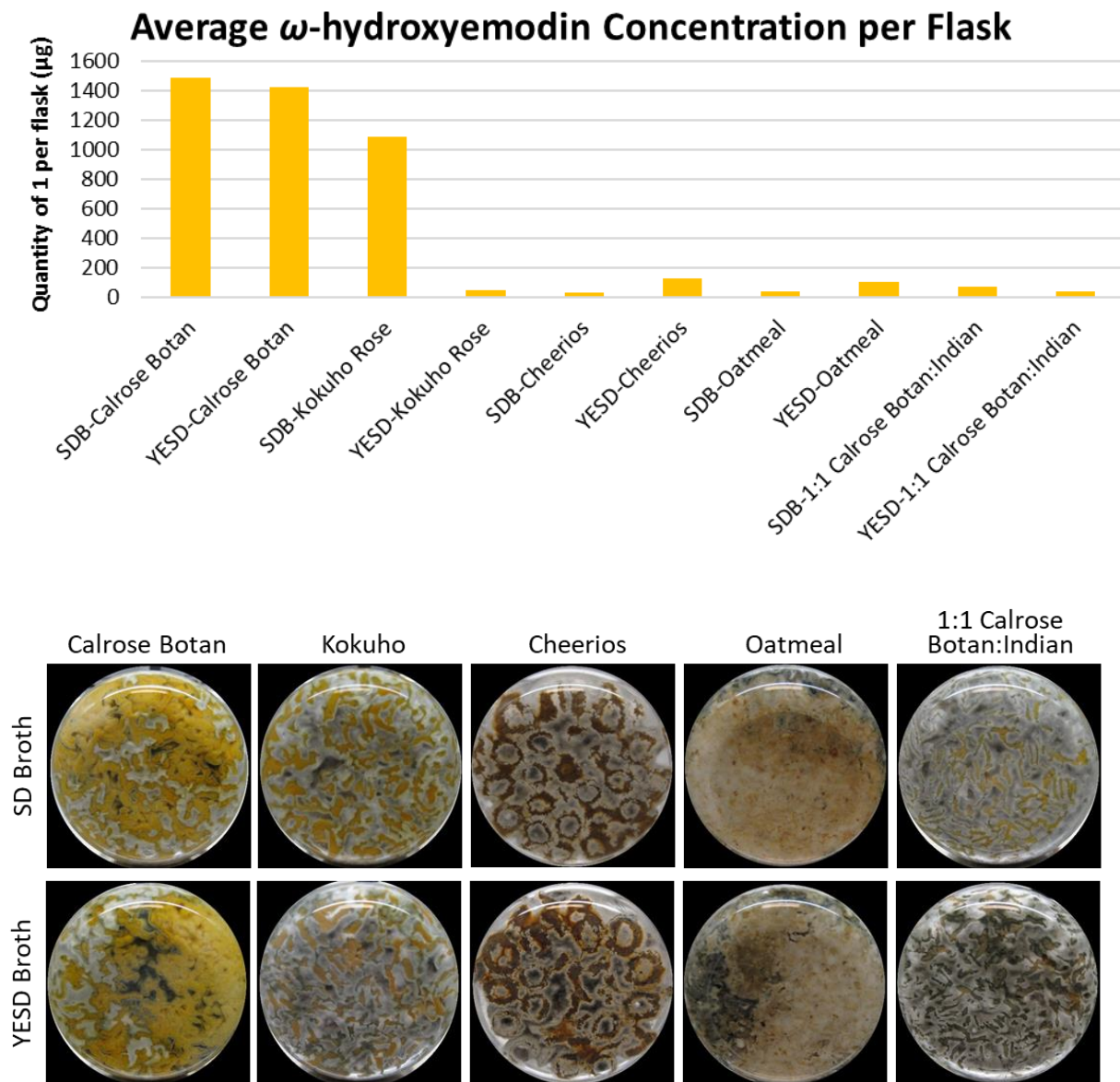
**Solid/Agar Cultures**



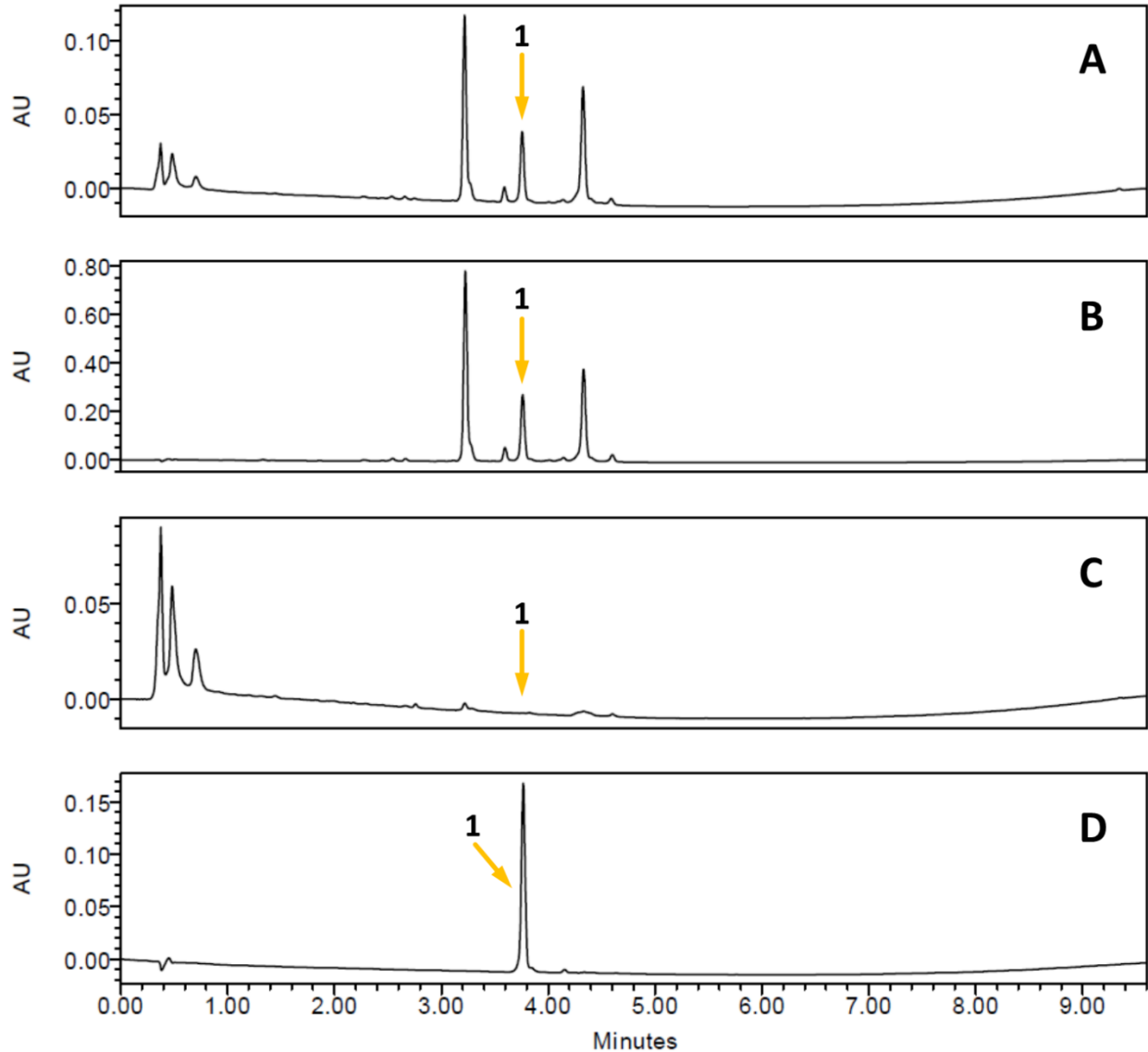
**Fig. 3S.** *Penicillium restrictum* (strain G85) on commercial rice (variety: Calrose Botan) using Sabouraud dextrose broth (SD broth; right) and YESD broth (left) for generation of the seed cultures. The amount of **1** isolated per flask was higher when SD broth was used for the seed culture (see Fig. 4S, Supporting Information). This increased amount of **1** was also apparent visually, as higher production of the polyhydroxyanthraquinones correlated to a darker orange/red coloration of the culture.



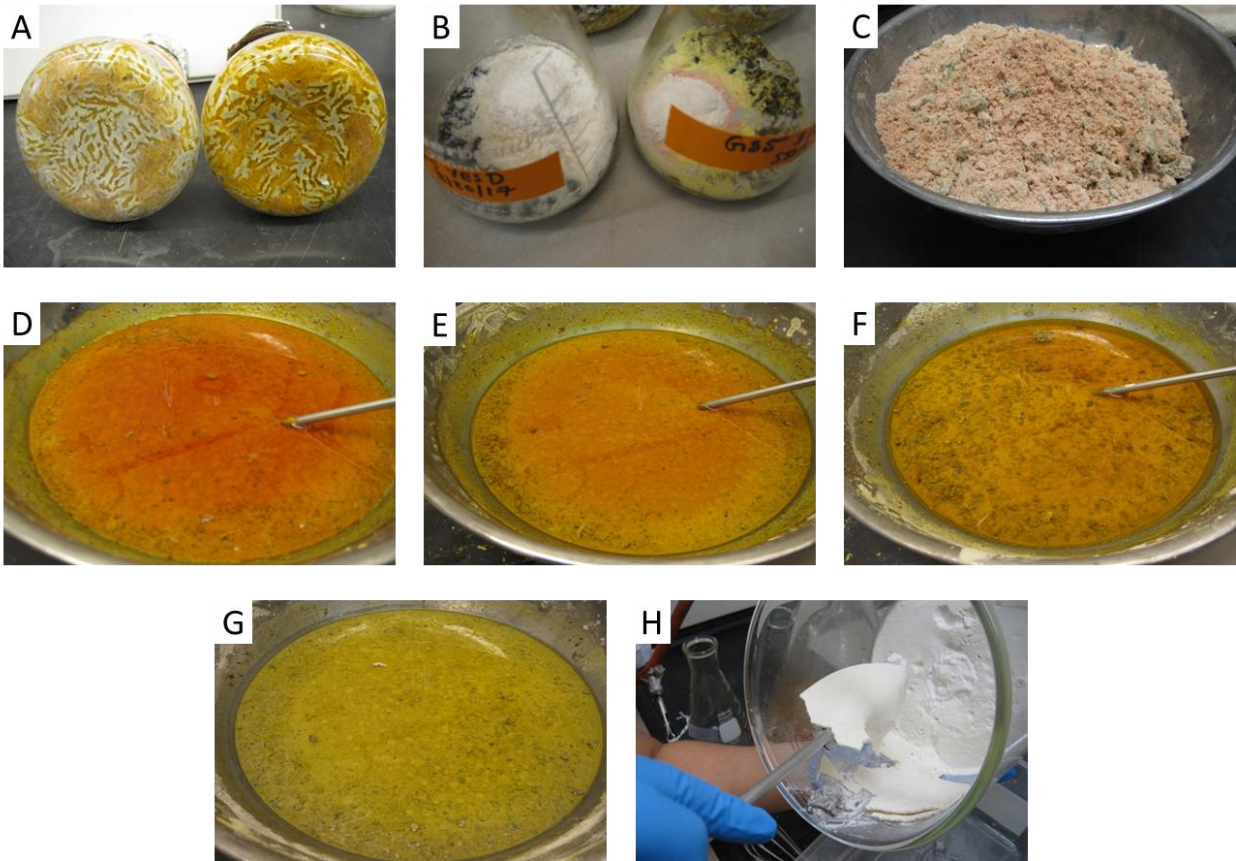
**Fig. 4S.** Quantity of  $\omega$ -hydroxyemodin (**1**) obtained after 4 wk growth on various media. *Penicillium restrictum* (strain G85) was grown for 4 wk on 5 different solid media: Calrose Botan rice, Kokuho Rose rice, Cheerios, oatmeal, and Calrose Botan:Indian (Udupi Sona Masoori) rice mixture. Each of these 5 solid media used one of 2 different seed cultures, Sabouraud dextrose broth (SD Broth) or yeast extract soy dextrose (YESD) broth. Each condition was grown in duplicate, and their concentrations were averaged. The amount of **1** per flask was determined by UPLC and is shown in the plot. Images of the bottoms of the flasks for each of the growth conditions is shown with the deepness of orange/red coloration correlating to the quantity of  $\omega$ -hydroxyemodin observed in each flask.



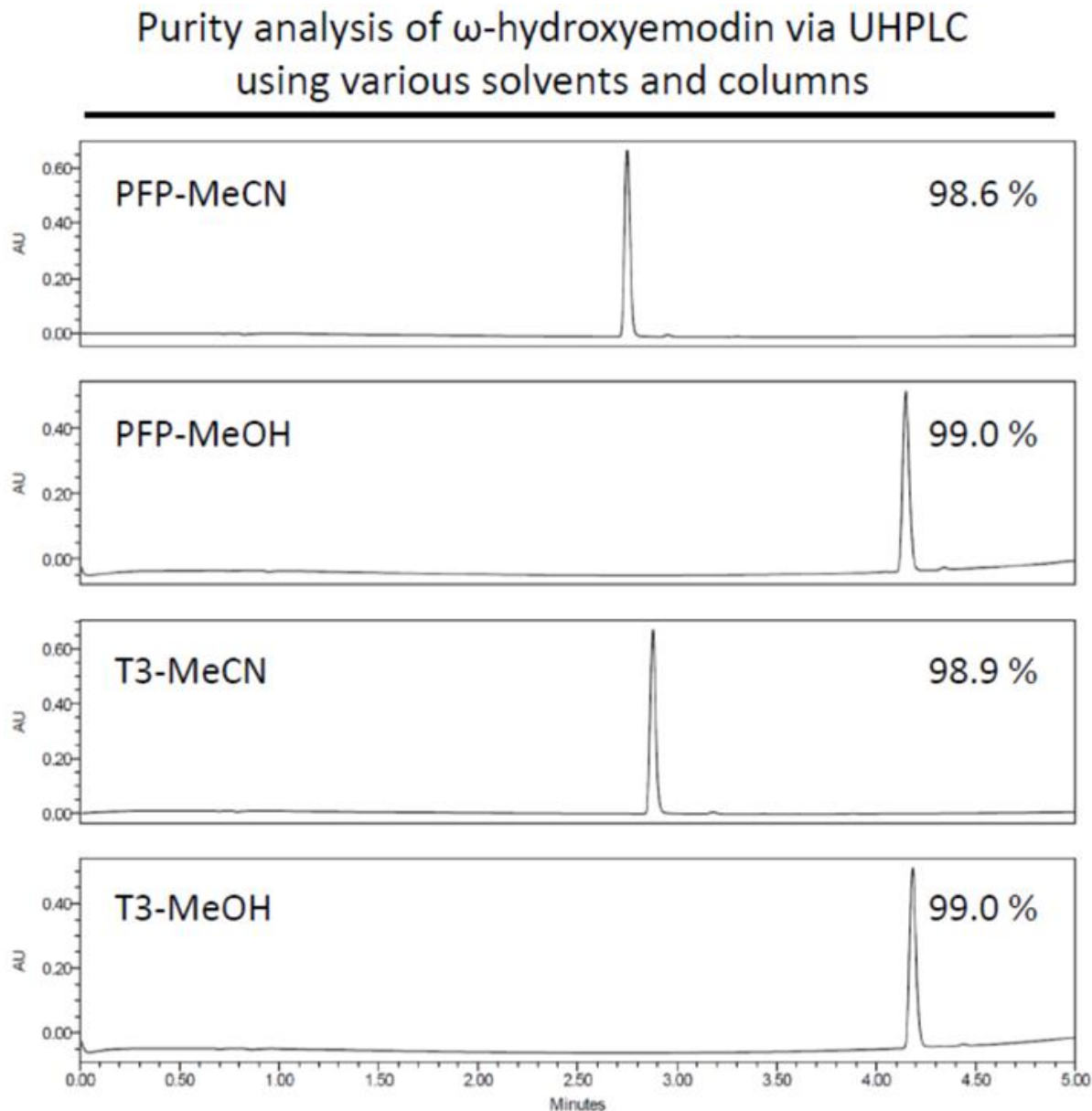
**Fig. 5S.** Recovery of  $\omega$ -hydroxyemodin (**1**) from the aqueous phase of a  $\text{CHCl}_3$  partition. A) Residual **1** observed in the aqueous layer of a typical  $\text{CHCl}_3$  partition. B) Chromatogram of the ethyl acetate layer showing the polyhydroxyanthraquinones recovered from the aqueous layer by partitioning with ethyl acetate. C) Chromatogram of the remaining aqueous layer after partitioning with ethyl acetate indicating the absence of **1** (and other polyhydroxyanthraquinones) in the aqueous layer. D) Chromatogram of purified **1**.



**Fig. 6S.** Stages in generating the initial extract. A) YESD broth (left) and SD broth (right) inoculated cultures. B) YESD broth (left) and SD broth (right) inoculated cultures with Celite added. C) Numerous SD broth cultures mixed with Celite in a mixing bowl. D) First round of acetone extraction. E) Second round of acetone extraction. F) Third round of acetone extraction. G) Fourth round of acetone extraction. H) Sugary materials captured upon passing through open silica column.



**Fig. 7S.** Evaluation of the purity of a batch of  $\omega$ -hydroxyemodin (**1**) obtained using the methods discussed in this paper.



**Columns:**

Waters Acquity UPLC HSS-PFP, 1.8  $\mu$ m, 100 x 2.1 mm column

Waters Acquity UPLC HSS-T3, 1.8  $\mu$ m, 100 x 2.1 mm column

**Conditions:**

Flow rate of 0.4 mL/min of the respective solvents, at a column temperature of 40 °C.

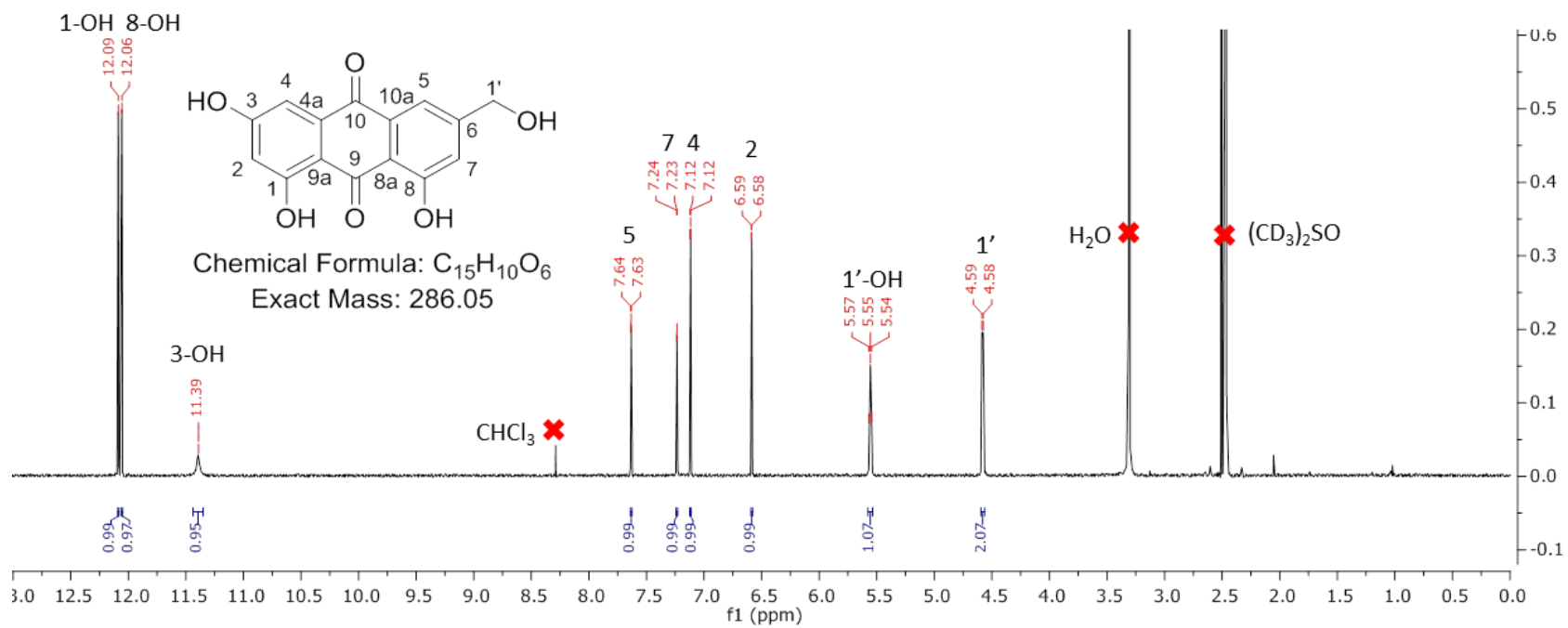
Injection volume of 3  $\mu$ L.

Observed at 288 nm.

**Gradients:**

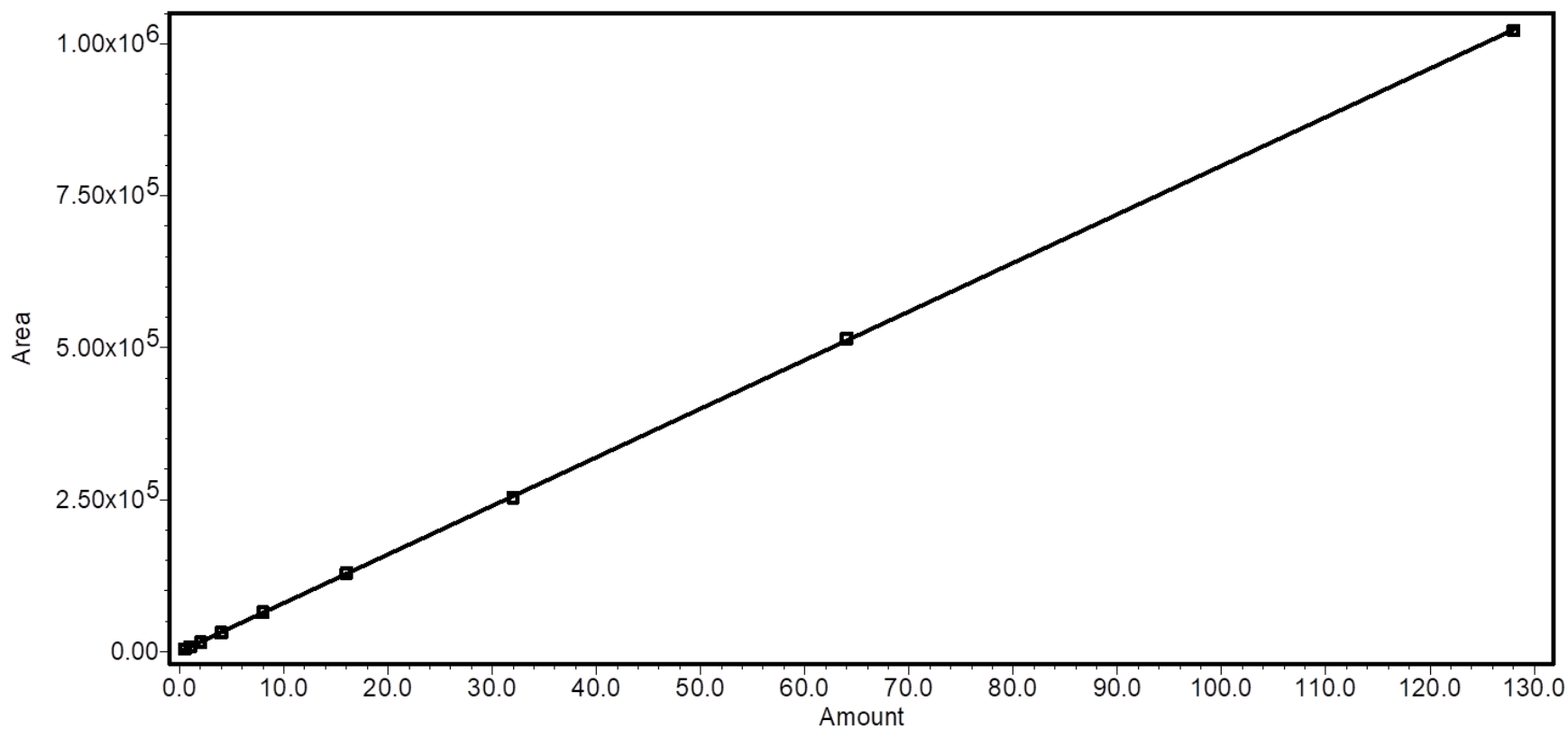
10:90 to 100:0 MeCN:water (0.1% formic acid) over 4.5 min.

Or 10:90 to 100:0 MeOH:water (0.1% formic acid) over 4.5 min.

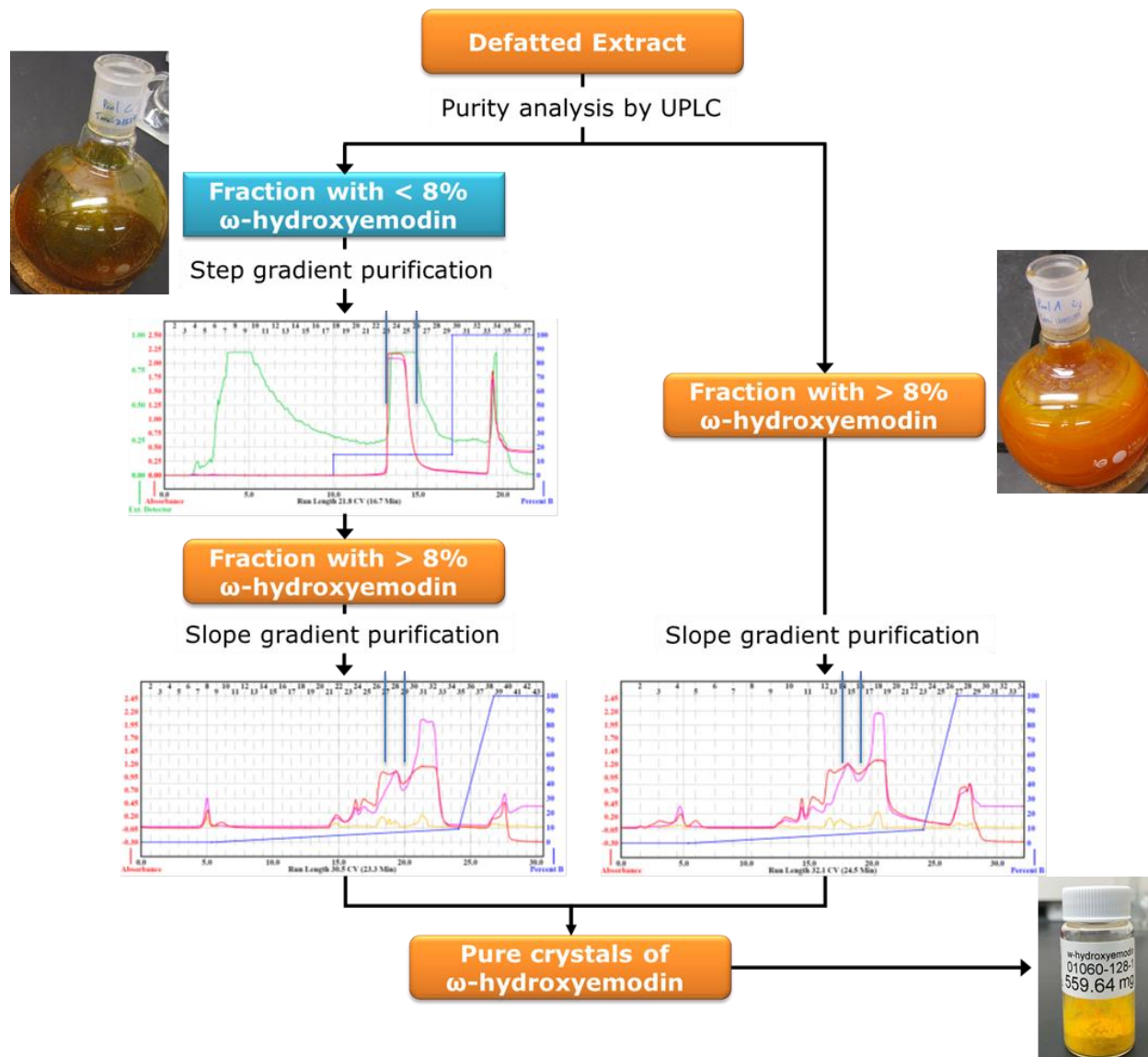


**Fig. 8S.**  $^1\text{H}$  NMR spectrum of  $\omega$ -hydroxyemodin (1) evaluated in  $\text{DMSO-}d_6$  on a 500 MHz instrument.

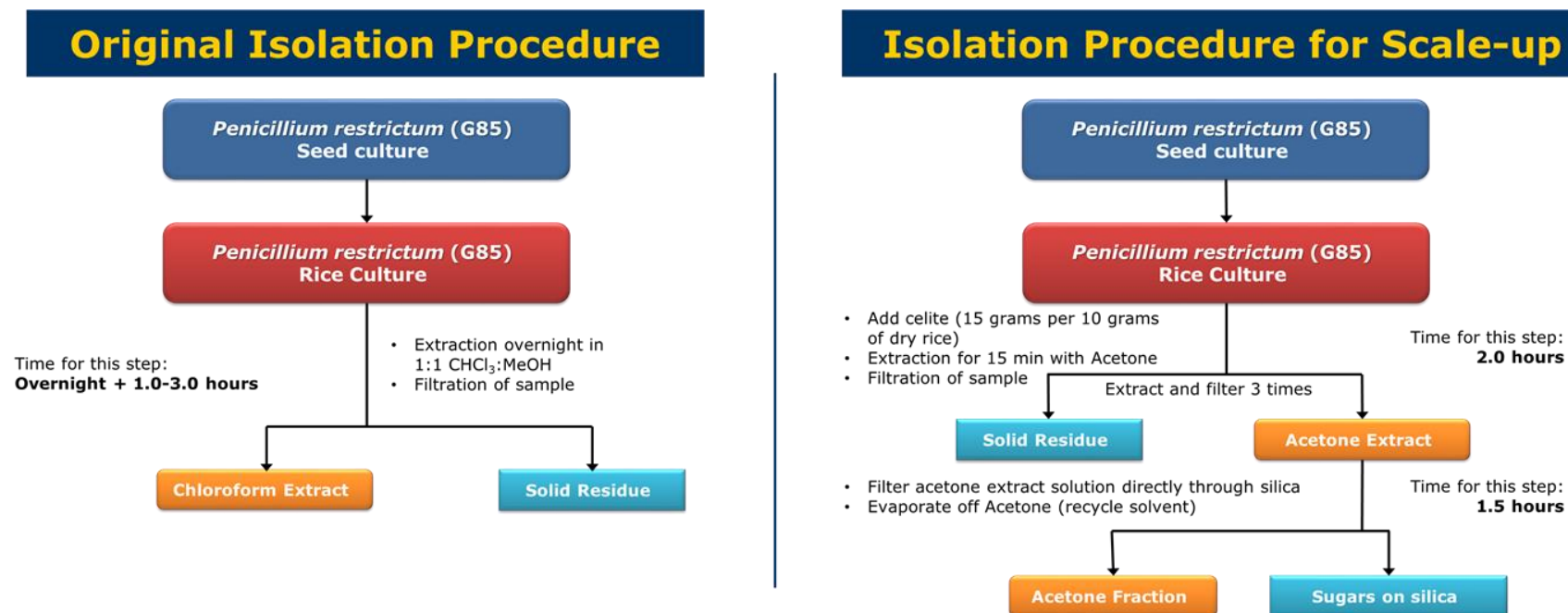
**Fig. 9S.** Single injection per point calibration curve for  $\omega$ -hydroxyemodin (**1**) in  $\mu\text{g}/\text{mg}$  of sample. Chromatograms were collected at 288 nm on an Acquity UPLC system using an Acquity BEH-shield column ( $2.1 \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) running a gradient elution from 10% ACN in  $\text{H}_2\text{O}$  (0.1% formic acid) to 100% ACN over 4.5 min at a flow rate of 0.4 mL/min and a column temperature of  $40^\circ\text{C}$ . Equation:  $Y = 7.99 \times 10^3 X + 2.72 \times 10^2$ ,  $R^2 = 0.99998$



**Fig. 10S.** Initial fractionation of G85 extract depending on  $\omega$ -hydroxyemodin (**1**) content. The bracketed fractions in each chromatogram are where **1** eluted. In the bottom 2 chromatograms, **1** formed crystals in the tubes upon elution. The observation of a crystalline precipitate when a fraction had  $>8\%$  **1** led to the development of a concentration procedure (left side of diagram), further facilitating the purification process.



**Fig. 11S.** Improvements in the extraction step of the purification of  $\omega$ -hydroxyemodin (**1**). Increasing the quantity of **1** recovered from the culture media.

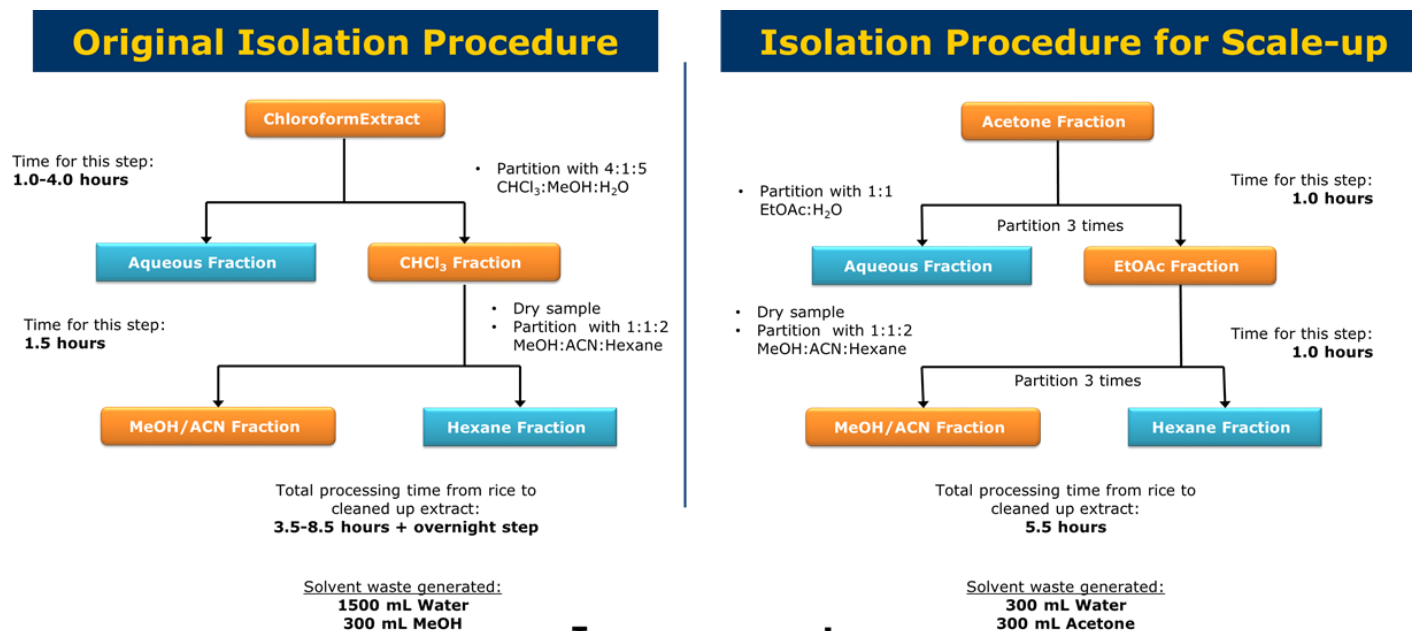


## Improvements

- Use greener Acetone vs. CHCl<sub>3</sub>
- No overnight step
- Higher compound recovery
- Consistent filtration time
  
- Silica step removes sugars and insolubles to improve later steps



**Fig. 12S.** Improvements in the partitioning steps of the purification of  $\omega$ -hydroxyemodin (**1**), serving to increase the recovery and purity of **1** through the process.



## Improvements

- Use greener EtOAc vs. CHCl<sub>3</sub>
- No emulsions
- Faster partition
- Higher compound recovery
- Consistent partition time
  
- Faster partition (due to lower volumes)
- Higher compound recovery
- Consistent partition time

## Overall effect

- Faster
- Higher yield
- Consistent processing time
- Easily scalable
- Greener solvents
- Less solvent waste

**Protocol 1S.** Example extraction procedure for the isolation of  $\omega$ -hydroxyemodin (**1**) rich extracts.

For this protocol discussion, a sample of 12 small scale samples will be used to show the workflow and about how much solvent is used at each step for a sample of this size. **Fig. 6S-A** is a photo of a YESD broth and an SD broth culture side by side

- 1) Add 2 scoops (~15 grams) of Celite to each of the 12 small scale flasks (15 grams Celite/ 10 g dry weight of rice) (**Fig. 6S-B** shows a YESD broth and an SD broth culture side by side with the Celite added).
- 2) Stir in the Celite and then transfer the Celite/rice mixture to a large stainless-steel mixing bowl (**Fig. 6S-C**).
- 3) Add 1000 mL of acetone, stir the mixture (adding solvent until you have a pancake batter consistency), and allow to soak for 15 min (**Fig. 6S-D**).
- 4) Filter the sample on a 240 mm Buchner funnel using Whatman type 1 qualitative filter paper, rinsing the solids with 200 mL of acetone.
- 5) Transfer the solids back into the mixing bowl, add 900 mL of acetone, and allow to soak for 15 min (**Fig. 6S-E**).
- 6) Filter the sample on a 240 mm Buchner funnel, rinsing the solids with 200 mL of acetone.
- 7) Repeat steps 5 and 6 1 more time using 800 mL of acetone (**Fig. 6S-F and Fig. 6S-G**). Note the sample in the photos was complete after 3 rounds of extraction as there is little obtained from a fourth round.
- 8) Prepare a rough silica column using a glass fritted filter funnel (course frit), ~800 mL of silica, and a large filter flask.
- 9) Pre-wet the silica with acetone and produce a level bed, then place a watch-glass on the surface of the silica (concave side up).
- 10) Combine the acetone filtrates from steps 4 through 7 and transfer the solution into a separatory funnel and dispense the sample slowly onto the surface of the watch-glass from step 9 (dispensing onto the watch glass prevents disturbance of the silica surface).
- 11) Apply vacuum as needed to draw the sample through the silica, eventually passing the entire combined acetone sample through the silica.
- 12) Wash the silica with fresh acetone until little color elutes from the silica (the silica should have trapped insoluble material on the surface and sugars/very polar compounds absorbed). Examination of the silica will show that even upon drying, the silica near the

top surface is gooey and in some cases will be a rubbery material similar to fondant (**Fig. 6S-H**).

- 13) Dry the acetone effluent (much less dark and cloudy after steps 10–12) with a moderate vacuum (~120 mBar) and recover the acetone for reuse. Continue to dry the sample under strong vacuum until either dryness or all trace of acetone has been removed (remaining acetone will interfere with the following partition step).
- 14) Add water to a volume of ~250 mL (or dry to this volume in step 13) and add 250 mL of ethyl acetate.
- 15) Stir together the solvents and place in a separatory funnel for ~15 min to allow the layers to separate (layers will not separate if acetone remains after step 13).
- 16) Drain the lower aqueous layer back into the original flask and collect the ethyl acetate layer.
- 17) Add 200 mL of fresh ethyl acetate to the aqueous layer and partition again using steps 15 and 16.
- 18) Partition the aqueous layer 1 or more additional times using 150 mL of fresh ethyl acetate each time until the ethyl acetate layer stops obtaining much color (typically 3 or 4 rounds total).
- 19) The combined ethyl acetate layers is dried completely (collecting the ethyl acetate for reuse).
- 20) The ethyl acetate partition product is resuspended in 100 mL of hexanes and 100 mL of 1:1 ACN:MeOH, mixed together and allowed to separate in a separatory funnel for 5 min.
- 21) The lower 1:1 ACN:MeOH layer is collected and 75 mL of fresh 1:1 ACN:MeOH added to the remaining hexanes layer, mixed, and allowed to settle for 5 min.
- 22) Step 21 is repeated for a third time using 50 mL of 1:1 ACN:MeOH. The hexanes layer should only be a mild yellow color due to fats content.
- 23) The combined 1:1 ACN:MeOH partition products are dried, weighed, and a 1 mg/mL sample prepared for UPLC analysis to determine its  $\omega$ -hydroxyemodin content.