# Isolation of Antirhinoviral Sesquiterpenes from Ginger (Zingiber officinale)

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# ISOLATION OF ANTIRHINOVIRAL SESQUITERPENES FROM GINGER (*ZINGIBER OFFICINALE*)

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ABSTRACT.—The dried rhizomes of Indonesian ginger, *Zingiber officinale*, were investigated for antirhinoviral activity in the plaque reduction test. Fractionation by solvent extraction, solvent partition, and repeated chromatography guided by bioassay, allowed the isolation of several sesquiterpenes with antirhinoviral activity. The most active of these was β-sesquiphellandrene [**2**] with an $IC_{50}$ of 0.44 μM vs. rhinovirus IB in vitro.

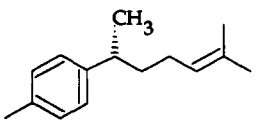
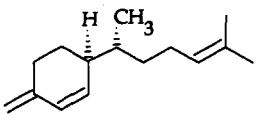
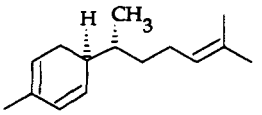
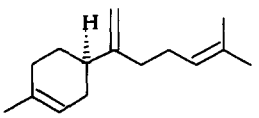
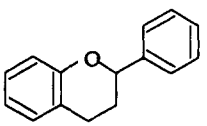
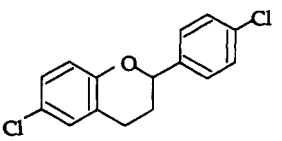
For some time higher plants have been investigated for antiviral activity and in particular for activity against rhinoviruses, which are among those viruses causing the common cold (1,2). Antirhinoviral activity appears to be particularly well established within the flavonoid series of natural products from plants. In addition, there has been synthetic work that has extended the range of flavonoid compounds with such activity and this has culminated in the discovery of the lipophilic 4′,6-dichloroflavan [**6**] (see Table 1) with an $IC_{50}$ of 0.007 μM vs. rhinovirus IB (RVIB) in vitro (3). The question arose as to whether other natural products, possibly of a lipophilic nature, might be found to have antirhinoviral activity. We became interested in ginger, *Zingiber officinale* Roscoe (Zingiberaceae), as a potential source of antirhinoviral agents, as it is readily available and is known to contain lipophilic secondary metabolites (4).

The use of ginger as a spice is well established and it has been prized as a medicinal agent since ancient times (4). Recently, however, its constituents have been investigated by modern pharmacological techniques. For example, MeOH extracts have been fractionated and inotropic effects on isolated guinea pig atria associated with gingerols (5). Also, using the effects of ginger on HCl/EtOH induced gastric lesions in rats, ar-curcumene [**1**], β-sesquiphellandrene [**2**], and β-bisabolene [**4**] among other compounds, have been identified as anti-ulcer agents (6). However, there appear to be no reports of ginger being fractionated for antiviral activity in the literature.

Preliminary experiments showed that a large portion of the antirhinoviral activity of a MeOH extract of Indonesian ginger rhizomes passed into the hexane phase after EtOAc-$H_2O$ and hexane-MeOH-$H_2O$ (10:9:1) partitions. Because of this it was decided to concentrate on the activity in a hexane extract and the final form of the bioassay-guided large-scale fractionation is shown in Scheme 1. The isolated components ar-curcumene [**1**], β-sesquiphellandrene [**2**], α-zingiberene [**3**], and β-bisabolene [**4**] were identified from the ir (7–10), nmr (7–9, 11–14) and ms (6,8,13,15) data. Crude fractions and compounds **1–4** were screened against RVIB in the plaque reduction test (2,3,16,17). For the crude fractions, the activities calculated from the dose-response curves are shown in Scheme 1, while the activities for compounds **1–4** and flavan **5**, the positive control, are given in Table 1.

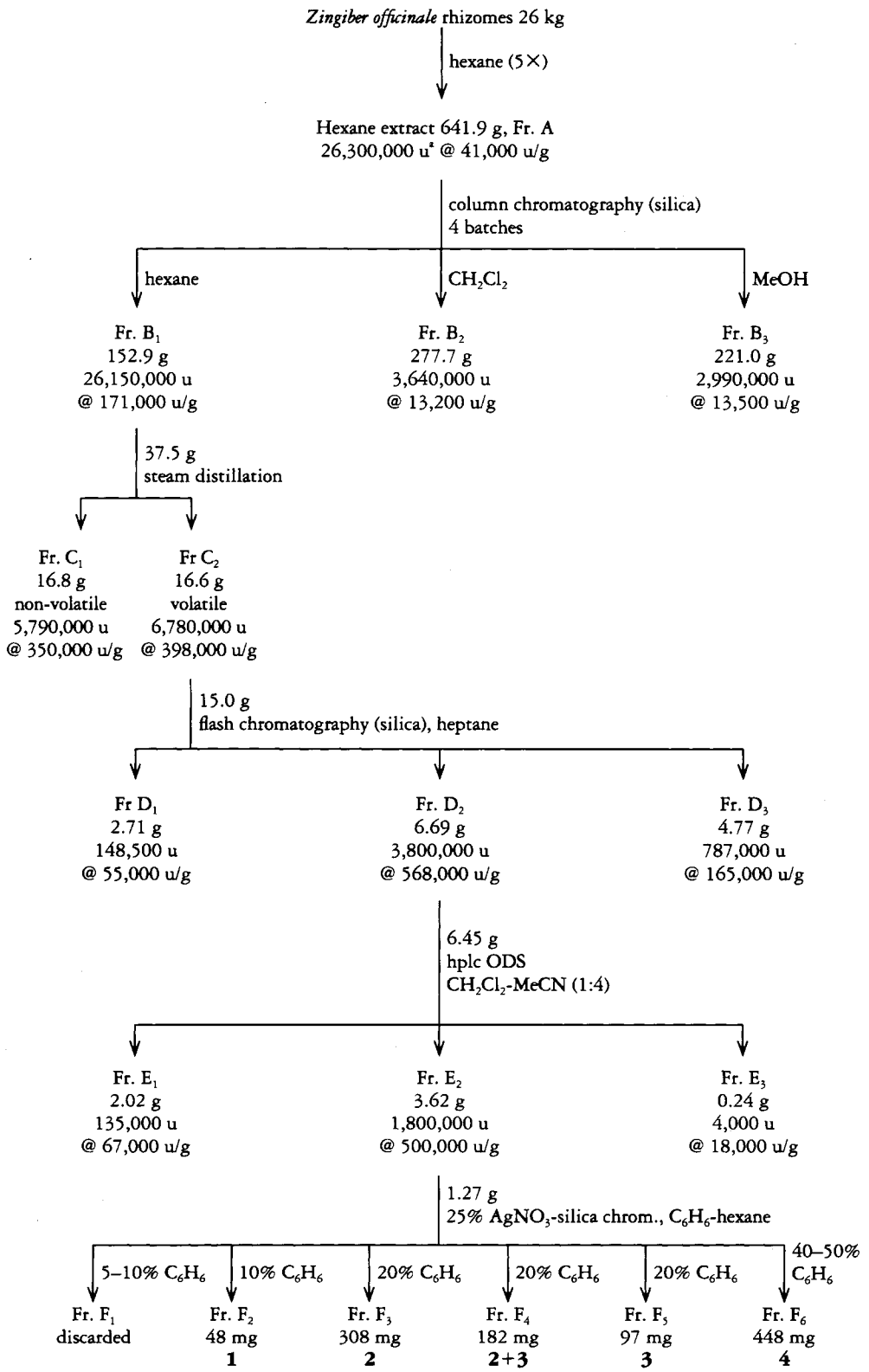
In order to use the plaque reduction test effectively it is important to distinguish between virally induced plaques and cytopathological effects due to sample toxicity. The visible toxic effects can vary between cell destruction and slight alter-

TABLE 1. Inhibitors of Rhinovirus IB In Vitro.

| | Compound | WRL cpd. no. | Glc peak no. | $ED_{50}$[a,b] |
|---|---|---|---|---|
| 1 |  ar-curcumene | BW20C85 | 1 | 20.4±7.3[c] |
| 2 |  β-sesquiphellandrene | BW333C85 | 4 | 0.90±0.10[c] |
| 3 |  α-zingiberene | BW331C85 | 2 | 1.90±0.41[c] |
| 4 |  β-bisabolene | BW332C85 | 3 | 14.3±1.59[c] |
| 5 |  flavan | | | 0.27±0.02[d] |
| 6 |  4′,6-dichloroflavan | BW683C77 | | 0.02±0.003 |

[a]The $ED_{50}$ is in μg per plate. Volume of overlay=10 ml.
[b]For compounds **1–5**, ± SEM, $n$=4; $ED_{50}$ of **6** calculated from data in Bauer *et al.* (3).
[c]No cytotoxicity observed up to top plate (20 μg/plate).
[d]No cytotoxicity observed up to top plate (1.35 μg/plate).

ation in the appearance of the cells (18). Care was taken to ensure that only points on the dose-response curve, at which samples incorporated into the overlay did not cause significant cytoxicity relative to cellular and viral controls, were used to calculate the $ED_{50}$. Sample concentrations were generally increased until cytotoxicity was evident and then the next dilution below this was used as the upper limit in the derivation of the $ED_{50}$. However, in the case of Fr. $B_2$ and Fr. $B_3$ (Scheme 1), slight toxicity, manifesting itself in a slight thinning of the cells, was seen at the highest concentrations, but in all other cases and in particular for compounds **1–5**, only non-toxic concentrations were used. For compounds **2–5**, the monolayers were completely cleared of plaques at the highest concentration (see Table 1).




SCHEME 1. Large-Scale Extraction of *Zingiber officinale* (Ginger) Rhizomes for Antirhinoviral Activity.

[a]For definition of 1 u, see Experimental.

Of the isolated sesquiterpenes, the most active was β-sesquiphellandrene [**2**], with an $IC_{50}$ of 0.44 μM. It is interesting to note the structural similarity between the sesquiterpenes **1–4**, flavan **5**, and 4′,6-dichloroflavan [**6**]; all the molecules are of a similar polarity and overall size. In particular, the potency of the sesquiterpenes is sensitive to small structural charges in the molecules (see Table 1). This sensitivity to structural features combined with potency in the μM range suggests action at a specific receptor site, as in the case of the antirhinoviral compounds WIN51711 and WIN52084 (19). Further work is needed to explore this and also to determine if the flavans and sesquiterpenes act at the same site.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—$^1$H-Nmr spectra were run on a Bruker AM-360FT spectrometer in $CDCl_3$ with TMS as internal standard at 360 MHz. Ir spectra were recorded as films between NaCl plates on a Perkin-Elmer 298 spectrometer. Low-resolution eims were obtained on a VG-7070 instrument operating at 70 eV. Accurate mass measurements were obtained on a Kratos MS90 instrument at 70 eV with perfluorokerosene ($m/z$=192.9888) as a reference mass. Tlc was carried out on Si gel plates (Merck, Si gel $GF_{254}$, 20 cm×5 cm, 0.2 mm thick, run over 10 cm). Zones were visualized by spraying with 1% vanillin/$H_2SO_4$ and then heating at 120°. Glc was carried out using a Pye-Unicam 104 gas chromatograph with fid and an OV101 (5% on Universal support, 2.1 m×4 mm i.d.) column at 130–220°. Analytical and prep. hplc were performed using a Dupont 8800LC System with uv detection at 236–240 nm; for analytical hplc Zorbax columns (25 cm×4.5 mm o.d.), with solvent flow 1 ml/min were used, and for prep. hplc, Zorbax columns (25 cm×5 cm o.d.) with solvent flow rate 16 ml/min were employed. Cc on Si gel was performed using Merck Si gel 60 (70–230 mesh). Flash cc on Si gel was carried out with Merck Si gel 60 (230–400 mesh). Large-scale evaporation of solvents was carried out using a Büchi Rotavapor 175 Ex (50 liter flask) with a coil condenser coupled to a refrigeration unit; the bath temperature was 30° and the coolant was circulated to the condenser at −20°; solvents were evaporated at water-pump vacuum. $C_6H_6$ and hexane for $AgNO_3$-Si gel chromatography were redistilled and dried over Na wire. Samples were routinely dried at 0.2–0.5 mm Hg at room temperature.

PLANT MATERIAL.—Commercial Indonesian ginger (*Zingiber officinale*) rhizomes were obtained from G. MacDonald & Co. Ltd., London, and identified by macroscopic appearance. Material for extraction was ground to a coarse powder by the Wellcome Foundation, Dartford, UK, before use.

BIOLOGICAL ASSAYS.—Antiviral activity was assayed in vitro by the plaque reduction test using confluent layers of M-HeLa cells (2,3,16,17). The assay was performed in 60 mm tissue culture plates which were seeded with $10^6$ cells in Eagle's minimum essential medium (MEM), containing 10% fetal calf serum (FCS). After the cells had reached confluence (overnight), the medium was decanted and the cells infected with RVIB in MEM for 1 h at 100–200 pfu per plate; excess virus was then decanted and replaced with the test sample incorporated in a mixture of MEM, FCS, tryptose broth, and agarose. For incorporation, samples were dissolved in EtOH and serial doubling dilutions made in EtOH; 100 μl sample dilutions were added into overlay such that the concentration of EtOH was no greater than 1% in the assay. The agarose overlay was left to gel, and the plates were incubated at 33° for 72 h. At the end of the incubation period, the monolayer was fixed with formol-saline, washed with $H_2O$, and stained with methyl violet. The plaques showed up as clear areas on a blue background. For each sample, plaque reductions were measured relative to virus controls; cell controls were set up without virus and without test sample. Using plaque reductions ($n \geq 3$), generally at non-toxic sample levels (see main text), for crude fractions an $ED_{50}$ (μg per plate) was derived from the dose-response curve and from this the specific activity in units/g, where one unit of activity (1 u) was defined as the activity that reduced the number of plaques by 50% as compared to the virus controls. Similarly, for the isolated components, dose-response curves allowed the calculation of $ED_{50}$s. Flavan **5** was used as the positive control in the plaque reduction test.

FRACTIONATION AND ISOLATION.—Ground Indonesian ginger rhizomes (26 kg) were extracted with hexane (5×180 liters) by stirring overnight at room temperature. The hexane extract was evaporated to a brown oil (Fr. A) which was subjected to cc on Si gel, with the fraction which eluted with hexane (colorless oil, Fr. $B_1$) being further processed by steam distillation. Volatile material (Fr. $C_2$) exhibited 3 zones at $R_f$ 0.70, 0.62 (most intense), and 0.52 on tlc (heptane; Si gel) and was further refined by flash cc and non-aqueous reversed-phase hplc. At this stage, the most active fraction (Fr. $E_2$) was essentially homogeneous by tlc ($R_f$ 0.62 with heptane; Si gel) and analytical hplc (both normal phase with silica and reversed-phase with ODS silica). When this fraction was examined by analytical glc (4,20) four

components were revealed and these were separated by cc using $AgNO_3$-impregnated Si gel (25% w/w) as the absorbent and a $C_6H_6$/hexane gradient as the eluent (6,7,11,21) to give Fr. $F_2$, Fr. $F_3$, Fr. $F_5$, and Fr. $F_6$.

*Fraction $F_2$, ar-curcumene* [**1**].—Colorless oil: 48 mg.

*Fraction $F_3$, β-sesquiphellandrene* [**2**].—Colorless oil: 308 mg.

*Fraction $F_5$, α-zingiberene* [**3**].—Colorless oil: 97 mg.

*Fraction $F_6$, β-bisabolene* [**4**].—Colorless oil: 448 mg.

Identifications of these isolates were performed as described in the text.

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