J Ind Microbiol Biotechnol (2003) 30: 721–731
DOI 10.1007/s10295-003-0101-x

ORIGINAL PAPER

**Sheo B. Singh · Hiranthi Jayasuriya · Raymond Dewey
Jon D. Polishook · Anne W. Dombrowski
Deborah L. Zink · Ziqiang Guan · Javier Collado
Gonzalo Platas · Fernando Pelaez · Peter J. Felock
Daria J. Hazuda**

Isolation, structure, and HIV-1-integrase inhibitory activity of structurally diverse fungal metabolites

Received: 9 June 2003 / Accepted: 1 November 2003 / Published online: 9 January 2004

Abstract HIV-1 integrase is a critical enzyme for replication of HIV, and its inhibition is one of the most promising new drug strategies for anti-retroviral therapy, with potentially significant advantages over existing therapies. In this report, a series of HIV-1 inhibitors isolated from the organic extract of fermentations from terrestrial fungi is described. These fungal species, belonging to a variety of genera, were collected from throughout the world following the strict guidelines of Rio Convention on Biodiversity. The polyketide- and terpenoid-derived inhibitors are represented by two naphthoquinones, a biphenyl and two triphenyls, a benzophenone, four aromatics with or without catechol units, a linear aliphatic terpenoid, a diterpenoid, and a sesterterpenoid. These compounds inhibited the coupled and strand-transfer reaction of HIV-1 integrase with an IC_{50} value of 0.5–120 μM. The bioassay-directed isolation, structure elucidation, and HIV-1 inhibitory activity of these compounds are described.

Keywords HIV-1-integrase inhibitors · Structurally diverse natural products · Fungal metabolites · Polyketides

Abbreviations *CH_3CN* Acetonitrile · *TFA* Trifluoroacetic acid · *CD_3CN* Deuterio acetonitrile · *CD_2Cl_2* Deuterio methylene chloride · *MEK* Methyl ethyl ketone · *ZnSe* Zinc selenide · *UV* Ultraviolet · *IR* Infrared · *NMR* Nuclear magnetic resonance · *COSY* Homonuclear H-H correlation spectroscopy · *HMQC* Heteronuclear multiple-quantum coherence · *HMBC* Heteronuclear multiple-bond correlation · *EIMS* Electron-impact mass spectrometry · *HREIMS* High-resolution electron impact mass spectrometry · *ESIMS* Electrospray ionization mass spectrometry · *HRFABMS* High-resolution fast atom bombardment mass spectrometry · *MES* 2-(*N*-morpholino)-ethane sulfonic acid

S. B. Singh (✉) · H. Jayasuriya · R. Dewey · J. D. Polishook
A. W. Dombrowski · D. L. Zink · Z. Guan
Natural Products Chemistry, Merck Research Laboratories,
P. O. Box 2000, Rahway, New Jersey 07065, USA
E-mail: sheo_singh@merck.com
Fax: +1-732-5946880

J. Collado · G. Platas · F. Pelaez
CIBE, Merck Sharp and Dohme de España,
S. A. Josefa Valcárcel 38, 28027 Madrid, Spain

P. J. Felock · D. J. Hazuda
Department of Antiviral Research,
Merck Research Laboratories, West Point,
Pennsylvania 19486, USA

Introduction

HIV-1 integrase is one of the three enzymes that are critical for viral replication. It catalyzes three essential steps: (1) assembly, (2) endonucleolytic cleavage (3′-end processing) of the viral DNA, and (3) strand transfer of the viral DNA into the host cell DNA [11, 32, 33, 34]. The other two key enzymes are reverse transcriptase and protease. The development and therapeutic administration of inhibitors of the latter two enzymes has had an enormous impact on controlling the spread of HIV-1 infection. However, the emergence of multi-drug-resistant virus, even in drug-naïve patients, has become a serious cause for concern, and anti-HIV-1 therapy with a new mode of action is needed. HIV-1 integrase is absent in host cells and is required for viral replication. Inhibition of integration is one of the most suitable viral targets and could lead to a completely new mode of therapeutic action using non-toxic agents. Recently, much progress has been made in the identification of inhibitors of this enzyme [12, 22, 37, 65, 68].

Natural products have been a very good source of novel inhibitors for many biological targets, most importantly anti-infective targets. We have screened over 200,000 organic extracts of natural products derived from over 50,000 prokaryotic and eukaryotic strains and over 3,000 plants collected from all continents represented by

South America (28.4%), Asia (24.2%), Europe (23%), Africa (17.5%), North America (4.4%), and Oceania (2.4%). All biological material was obtained from various worldwide locations with strict accordance with the Rio Convention on Biodiversity. Screening was carried out using recombinant HIV-1 integrase (50-220 AA) initially in the coupled-assay format [21] and subsequently employing the strand-transfer format [21]. This led to the discovery of a variety of natural product inhibitors of HIV-1 integrase, including equisetin [21, 46], integric acid [47, 48], complestatins [49], integracins [50], integrastatins [51], integramycin [52], integramides [53], integracides [54], cytosporic acid [23], and chaetochromins [55]. Continued screening of the fungal extracts led to the discovery of a number of other novel and known natural products. In this report, the bioassay-directed isolation, structure elucidation, and HIV-1 inhibitory activity of these compounds are described.

Materials and methods

General

Reagents and chemicals were obtained from Sigma-Aldrich unless otherwise stated. All solvent extracts were dried over anhydrous Na_2SO_4. NMR spectra were recorded on Varian Inova 400 or 500 MHz instruments operating at 400 and 500 MHz for ^1H and 100 and 125 MHz for ^{13}C nuclei. An HP1100 was used for analytical HPLC. LC-MS was carried out on a Thermo Quest LCQ instrument using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Mass spectra were recorded on a JEOL SX-102A (electron impact, EI, 90 eV). High-resolution mass spectral analyses were done either on a Thermo Quest FTMS using electrospray ionization or a JEOL SX-102A using perfluorokerosenes (PFK) as internal standards. For column chromatography, silica gel H (E. Merck 60–200 mesh) was used. Five types of production media were used:

- F1 medium (g/250-ml Erlenmeyer flask): Cracked corn (10), 20 ml of a solution containing (mg/l) ardamine PH (100), KH_2PO_4 (50), $MgSO_4{\cdot}7H_2O$ (50), sodium tartrate (50), $FeSO_4{\cdot}7H_2O$ (5), $ZnSO_4$ (5) [29]
- CYS80 medium (g/l): Sucrose (80), corn meal (50) and yeast extract (1) [39]
- MV8 medium (g/l): Maltose (75), V8 juice (200 ml), soy flour (1), L-proline (3), MES (16.2, pH 6.5) [13]
- BRFT medium (g/ 250-ml Erlenmeyer flask): Brown rice (10), 20 ml of a solution containing in (g/l) yeast extract (1), KH_2PO_4 (0.5), sodium tartrate (0.5) [40]
- AD2 vermiculite-based medium (g/l): Glucose (autoclaved separately) (150), glycerol (20), yeast extract (4), $NaNO_3$ (1), monosodium glutamate (3), Na_2HPO_4 (0.5), $MgSO_4$ $7H_2O$ (1), trace elements (1 ml), $CaCO_3$ (8), pH 7. The trace elements solution contains (g/l): $FeCl_3{\cdot}6H2O$ (5.8), $MnSO_4{\cdot}H_2O$ (0.1), $CoCl_2{\cdot}6H_2O$ (0.02), $CuSO_4{\cdot}5H_2O$ (0.015), $Na_2MoO_4{\cdot}2H_2O$ (0.012), $ZnCl_2$ (0.02), $SnCl_2{\cdot}2H_2O$ (0.005), H_3BO_3 (0.01), KCl (0.02), HCl (2 ml). The AD2 autoclaved solution (220 ml) was added to 675 ml (by volume) of previously sterilized large-particle vermiculite in a 2-l roller bottle, at the time of inoculation [13].

HIV-1 integrase assay

In the coupled assay, unprocessed donor DNA was incubated with the HIV-1 integrase and the inhibitor at the same time. In the strand-transfer assay, unprocessed DNA was substituted with preprocessed DNA and the reaction was repeated as in the coupled assay, except inhibitors were added after a washing step of excess integrase. In both assays, the final product measured was the result of the strand-transfer reaction. For coupled and strand-transfer HIV-1 integrase assays, see references [22, 21]. Briefly, the microtiter plate assay for strand transfer was carried out with an immobilized 30-bp U5 donor substrate and a 20-bp target substrate biotinylated at the 3′-end of each DNA strand. Each reaction mixture of 100 µl consisted of 20 mM HEPES (pH 7.8), 25 mM NaCl, 25 mM $MnCl_2$, 5 mM β-mercaptoethanol, 50 mg bovine serum albumin per ml, and integrase (50 nM final concentration). The final concentration of DMSO in all reactions was 10%. Integrase was assembled with immobilized LTR oligonucleotides for 30 min at 37 °C. For coupled assays, the strand.transfer reaction was initiated by the addition of 5 µl of the biotinylated target substrate (150 nM final concentrations). For the strand-transfer reaction, unbound enzyme was removed and the reaction and the reaction buffer were exchanged before addition of the target substrate. Strand-transfer reaction mixtures were incubated for 30 min at 37 °C. Strand-transfer products were detected using alkaline-phosphatase-conjugated avidin. IC_{50} values were determined using a series of two-fold dilutions in duplicate or triplicate starting at 100–200 µM. Representative data from at least two independent measurements are presented.

Fermentation of *Cylindrocarpon ianthothele* for the production of 8-*O*-methylanthrogallol (**1**)

The producing organism (MF6391) was identified as *Cylindrocarpon ianthothele* Wollenw. based on a combination of characteristics that included purple-colored colonies, macroconidia measuring 55.5×9.4 µm with rounded ends, and lack of chlamydospores and microconidia [9]. The fungus was isolated from a soil sample collected in Grand Basin (Mauritius Island), following a conventional dilution plating method [38], using a medium supplemented with cyclosporine as described in [39]. For production of the compound, seed flasks were prepared from fresh slants of the isolate MF6391 in potato dextrose agar (PDA, Difco) as described [23]. Two-ml portions of the resulting cultures were used to inoculate 250 ml unbaffled Erlenmeyer flasks containing 50 ml CYS80 medium [39], which were incubated at 25 °C in a rotatory shaker at 220 rpm for 21 days.

Extraction and isolation of 8-*O*-methylanthrogallol (**1**)

The fermentation broth (250 ml) of MF6391 was extracted with 300 ml MEK by shaking it on a shaker for 30 min. The MEK layer was separated and a 60-ml aliquot was concentrated under reduced pressure to give 100 mg of dark brown solid, which was chromatographed on a 1-l Sephadex LH20 column in methanol. Active fractions eluted in a broad zone (800–1,600 ml) were pooled and concentrated to produce 13.8 mg of a fraction highly enriched with **1**. The enriched fraction was chromatographed on a reversed-phase HPLC column (Zorbax RX C-8, 21×250 mm) and eluted with a 60-min gradient of 20–80% aqueous $CH_3CN + 0.1\%$ TFA at a flow rate of 8 ml/min. Compound **1** eluted between 27 and 32 min and upon storage at 4 °C provided 2.1 mg yellow-orange precipitate of 8-*O*-methylanthrogallol.

t_R 6.96 min (Zorbax RX C-8, 4.6×250 mm, 60% aqueous $CH_3CN + 0.1\%$ TFA, 1 ml/min). UV (methanol) λ_{max} 212 ($\epsilon = 40{,}800$), 248 (16,900), 283 (32,700), 420 (10,500) nm; IR (ZnSe) ν_{max} 3400, 2965, 1654, 1626, 1543, 1455, 1348, 1244, 1059, 1005, 790, 761, 640 cm^{-1}; ^1H NMR (acetone-d_6) 2.50 (3H, s, H_3-11), 4.01 (3H, s, OCH_3), 7.28 (1H, s, H-4), 7.37 (1H, s, H-7), 7.66 (1H, s, H-5), 13.5 (1H, s, OH-1); ^{13}C NMR (acetone-d_6) 22.6 (C-11), 57.2 (OCH_3), 108.7 (C-4), 112.7 (C-9a), 119.6 (C-8a), 120.1 (C-7), 121.6 (C-4), 137.0 (C-10a), 139.8 (C-2), 140.1 (C-4a), 148.4 (C-6), 151.7 (C-3), 152.8 (C-1), 162.4 (C-8), 182.8 (C-10), 188.9 (C-9); HREIMS (m/z) 300.0623 (M^+, calculated for $C_{16}H_{12}O_6$: 300.0634).

Fermentation of *Inonotus tamaricis* for the production of hispidin (**2**) and caffeic acid (**3**)

The producing organism *Inonotus tamaricis* was isolated from the internal tissues of a basidioma of *I. tamaricis* collected in Torrejón, Madrid (Spain), following the method of isolation of basidiomycetes from their fruit bodies described by Worrall [66]. For production of the compound, seed flasks were prepared from fresh slants of the isolate MF6944 in PDA as described in [39]. Two-ml portions of the resulting culture were used to inoculate a solid corn-based F1 medium in 250-ml Erlenmeyer flasks as described earlier [29]. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for 28 days.

Extraction and isolation of hispidin (**2**) and caffeic acid (**3**)

The fermentation broth (250 ml) of MF6944 was extracted with 300 ml MEK by shaking for 30 min. The MEK layer was separated and a 20-ml aliquot was concentrated under reduced pressure to give 50 mg of a dark-brown solid. The solid material was dissolved in a 1:1 mixture of methanol-tetrahydrofuran (1 ml) and chromatographed on a reversed-phase HPLC column (Zorbax RX C-8, 21×250 mm). The column was eluted with a 60-min gradient of 20–80% aqueous CH_3CN containing 0.1% TFA at a flow rate of 10 ml/min. Fractions eluting at 4–7 (fraction A) and 14 –18 min (fraction B) possessed most of the HIV-1-integrase inhibitory activity. Fraction A was rechromatographed on the same HPLC column but eluted with a 60-min gradient of 10–25% aqueous CH_3CN containing 0.1% TFA at a flow rate of 10 ml/min. The integrase inhibitory activity eluted at 27–34 (fraction C) and 47–53 (fraction D) min. The chromatographically identical fractions B and D were pooled, concentrated under reduced pressure, and lyophilized to give hispidin (10 mg, 500 mg/l) as a dark-brown powder. Fraction C was similarly concentrated to give caffeic acid (2.7 mg, 135 mg/l) as a dark-brown powder.

Hispidin (**2**): t_R 11.84 min (Zorbax RX C-8, 4.6×250 mm, 20–80% aqueous $CH_3CN + 0.1\%$ TFA in 20 min, 1 ml/min); UV λ_{max} (methanol): 371, 291, 251, 220, 207 nm; IR ν_{max} (ZnSe): 3200, 1657, 1606, 1545, 1442, 1369, 1258, 1197, 1152, 1116, 1057, 1033, 1018, 963 cm^{-1}; ^1H NMR (acetone-d_6) 5.3 (1H, d, $J = 2.0$ Hz, H-3), 6.15 (1H, d, $J = 2.0$ Hz, H-5), 6.67 (1H, d, $J = 16$ Hz, H-7), 7.25 (1H, d, $J = 16$ Hz, H-8), 7.02 (1H, dd, $J = 8.4$, 1.2 Hz, H-10), 6.86 (1H, d, $J = 8.4$ Hz, H-11), 7.15 (1H, d, $J = 1.5$ Hz, H-14), ^{13}C NMR (acetone-d_6) 171.0 (C-2), 89.9 (C-3), 170.0 (C-4), 100.2 (C-5), 160.1 (C-6), 116.8 (C-7), 134.8 (C-8), 127.9 (C-9), 120.7 (C-14), 115.5 (C-13), 146.9 (C-12), 145.3 (C-11), 113.8 (C-10). HREIMS (m/z): 246.0529 $[(M)^+$, (calculated for $C_{13}H_{10}O_5$: 246.0528).

Caffeic acid (**3**): t_R 7.30 min (Zorbax RX C-8, 4.6×250 mm, 20% aqueous $CH_3CN + 0.1\%$ TFA in 20 min, 1 ml/min).

Preparation of trimethyl ether of hispidin (**4**)

Hispidin (3 mg) was dissolved in 0.1 ml methanol and diluted with 0.5 ml methylene chloride. The solution was cooled to 0 °C, mixed with a methylene chloride solution of freshly prepared diazomethane (0.5 ml) was added, and stored overnight at 4 °C. Solvents were evaporated under a stream of nitrogen and the major product was purified by silica-gel preparative TLC (methylene chloride-methanol, 93:7). The major UV band was eluted with 10% methanol in methylene chloride to give 2.2 mg hispidin trimethyl ether (**4**) as a yellow powder.

^1H NMR (CD_2Cl_2) 3.82 (3H, s, OCH_3-4), 3.86 (3H, s, OCH_3-12), 3.88 (3H, s, OCH_3-13), 5.44 (1H, d, $J = 2$ Hz, H-3), 5.95 (1H, d, $J = 2$ Hz, H-5), 6.58 (1H, d, $J = 16$ Hz, H-7), 6.95 (1H, d, $J = 8.4$ Hz, H-11), 7.07 (1H, d, $J = 1.5$ Hz, H-14), 7.12 (1H, dd, $J = 8.4$, 1.2 Hz, H-10), 7.45 (1H, d, $J = 16$ Hz, H-9), EIMS (m/z) 288 (M^+).

Fermentation of *Xeromphalina junipericola* for the production of xerocomic acid (**5**)

The producing organism *Xeromphalina junipericola,* G. Moreno and Heykoop (Agaricales, Basidiomycetes) [6] was isolated from the internal tissues of a basidioma of *X. junipericola* growing on the dead wood of *Juniperus thurifera* collected in Torrejón, Madrid (Spain), using the procedure described by Worrall [66] for the isolation of basidiomycetes from their fruit bodies. Seed flasks were prepared from fresh slants of the isolate MF6945 in PDA as described [39]. Two-ml portions of the resulting culture were used to inoculate BRFT medium in 250-ml Erlenmeyer flasks [40]. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for 28 days.

Extraction and isolation of xerocomic acid (**5**)

A 25-ml broth culture of MF6945 was extracted with 25 ml MEK. The organic layer, containing HIV-1-integrase inhibitory activity, was separated and concentrated to dryness under reduced pressure to give 140 mg of a yellow semi-solid. This material was dissolved in a 5 ml of a 1:1 mixture of methylene chloride-methanol and chromatographed on a 1-l Sephadex LH20 column in methanol. The activity was highly retained and eluted in 1,000–1,900 ml elution volume. The pooled fractions were concentrated to give 4.3 mg of a yellow solid, which contained >85% xerocomic acid. This fraction was chromatographed on a Zorbax RX C-8 (9.4×250 mm) column and eluted at 4 ml/min with a 60-min gradient of 20–80% aqueous $CH_3CN + 0.1\%$TFA, initially holding at 20% CH_3CN for 5 min. The activity eluted in a single broad peak between 15 and 19 min. These fractions were pooled and lyophilized to give 2 mg xerocomic acid (**5**) as a yellow powder.

t_R 5.7 min (Zorbax RX C-8, 4.6×250 mm, 70% aqueous $CH_3CN + 0.1\%$ TFA, 1 ml/min); ^1H NMR (CD_3CN) δ 6.80 (2H, d, $J = 8.5$ Hz, H-3,5), 6.81 (1H, d, $J = 8.5$ Hz, H-5′), 7.11 (2H, d, $J = 8.5$ Hz, H-2,6), 7.57 (1H, dd, $J = 8.5$, 2.5 Hz, H-6′), 7.71 (1H, d, $J = 2.5$ Hz, H-2′); ^{13}C NMR (CD_3CN) δ 100.0 (C-5″), 114.9 (C-2″), 115.5 (C-3,5), 116.29 (C-5′), 118.6 (C-2″), 120.4 (C-6′), 125.3 (C-1′), 128.3 (C-1), 132.9 (C-2,6), 144.7 (C-4′), 145.3 (C-3′), 154.5 (C-4″), 157.7 (C-4), 161.2 (C-3″), 167.2 (C-1″), 169.6 (C-6″); ESIMS (m/z) 355 $(M\text{-}H)^{-1}$, HREIMS (m/z) 644.226 (calculated for $C_{18}H_{12}O_{8+}4\times$TMS: 644.2113).

Fermentation of *Penicillium* sp. for the production of deoxyfunicone (**6**)

The producing organism, a strain of *Penicillium* sp., was isolated from ash from Mount Pinotubo, Philippines. For production of the compound, seed flasks were prepared from frozen vegetative mycelia of the isolate as described [13]. Ten-ml aliquots of the seed culture were used to inoculate AD2 medium in 2-l roller bottles. The roller-bottle production vessels were incubated on a Wheaton roller machine at 22 °C and 70% relative humidity for 21 days.

Extraction and isolation of deoxyfunicone (**6**)

The fungal growth in a 2-l roller bottle above was extracted with 250 ml MEK by rolling on a roller machine for several hours. The organic layer was separated and a 60-ml aliquot was concentrated to dryness to afford 210 mg of a brown gum. The gum was dissolved in a 10 ml of a 1:1 mixture of methylene chloride:methanol and chromatographed on a 1-l Sephadex LH20 column eluted with methanol. The biological activity eluted in a 600–800 ml elution volume. The active fractions were combined and concentrated to yield 120 mg of a solid containing deoxyfunicone. Sixty mg of the solid was chromatographed on a reversed-phase HPLC column (Zorbax RX C-8, 21×250 mm) and eluted with a 40-min gradient of 20–80% aqueous $CH_3CN + 0.1\%$ TFA at a flow rate of 8 ml/min.

The HIV-1-integrase inhibitory activity eluted in fractions between 29 and 33 min; these were pooled and lyophilized to yield 12.3 mg of a fraction enriched with 85% deoxyfunicone as a buff powder. The enriched fraction was further purified by preparative silica-gel TLC (hexane-ethyl acetate, 3:2) to give pure deoxyfunicone (7.4 mg) as a buff powder.

t_R 8.25 min (Zorbax RX C-8, 4.6×250 mm, 50% aqueous CH_3CN + 0.1% TFA, 1 ml/min); HREIMS (*m*/*z*) 358.1073 (36%, M+, calculated for $C_{19}H_{18}O_7$: 358.1052). For ^1H and ^{13}C NMR spectra, see [44].

Fermentation of *Talaromyces flavus* for the production of altenusin (**7**)

The producing organism MF6943 was isolated from leaf litter collected in Bolondo meadows, Bata, Equatorial Guinea, using the particle filtration technique described by Bills and Polishook [8]. The strain was identified as *Talaromyces flavus* (Klöcker) Stolk and Samson (Eurotiales, Ascomycetes) [58] on the basis of its yellow gymnothecium containing ellipsoid and spinose ascospores, 3– μm in diameter, which originated from the club-shaped ascogonia with coiled antheridium [58, 41]. No anamorph was observed in the culture medium recommended for the identification of *Talaromyces* sp., namely Czapek yeast extract agar, malt extract agar, and 25% glycerol nitrate agar [41]. Seed flasks were prepared from the fresh slants of the isolate MF6943 in PDA as described [39]. Two-ml portions of the resulting culture were used to inoculate 250-ml unbaffled Erlenmeyer flasks containing 50 ml CYS80 medium, which were incubated at 25 °C in a rotatory shaker at 220 rpm for 7 days.

Extraction and isolation of altenusin (**7**)

Ten ml of fermentation broth of MF6943 were extracted with 20 ml MEK which was concentrated to dryness under reduced pressure to give 30 mg of a gum. The gum was dissolved in methanol and chromatographed on a 200-ml Sephadex LH20 column that was eluted with methanol. HIV-1-integrase inhibitory activity eluted in 310–400 ml elution volume, which was concentrated to afford 4.5 mg (450 mg/l) of pure altenusin.

t_R 7.11 min (Zorbax RX C-8, 4.6×250 mm, 40% aqueous CH_3CN + 0.1% TFA, 1 ml/min), ^1H NMR ($CDCl_3$-CD_3OD, 4:1) δ 1.85 (3H, s, CH_3), 3.73 (3H, s, OCH_3), 6.10 (1H, d, J = 2.4 Hz, H-4), 6.37 (1H, d, J = 2.4 Hz, H-6), 6.48 (1H, s, H-12), 6.54 (1H, s, H-9); ^{13}C NMR ($CDCl_3$-CD_3OD, 4:1) δ 18.9 (C-14), 55.3 (OCH_3), 99.7 (C-4), 110.1 (C-6), 115.4 (C-2, 12), 116.2 (C-9), 127.1 (C-13), 134.5 (C-8), 141.3 (C-7), 143.0 (C-10), 146.0 (C-11), 162.9 (C-5), 164.1 (C-3), 173.5 (C-1); HREIMS (*m*/*z*) 290.0834 (M^+, calculated for $C_{15}H_{14}O_6$: 290.0790).

Fermentation of *Aspergillus candidus* for the production of terphenyllin (**8**) and 3-hydroxyterphenyllin (**9**)

The producing organism was identified as *Aspergillus candidus* (MF6941) Link (Eurotiales anamorph, Ascomycetes) based on a combination of morphological characteristics that included the formation of white- or cream-colored conidial masses when the fungus was cultured on Czapek yeast extract agar or malt extract agar, with conidia born, on biseriate aspergilla characterized by having large vesicles almost totally covered by metulae [26]. The fungus was isolated from a sample of cow dung collected in Jorá Caves, Asturias (Spain), using the particle filtration method described by Bills and Polishook [8]. Two-ml portions of the seed culture of MF6941 prepared from the fresh slants of isolate [39] were used to inoculate 50 ml MV8 medium in 250-ml unbaffled Erlenmeyer flasks [13], which were incubated at 25 °C in a rotatory shaker at 220 rpm for 14 days.

Extraction and isolation of terphenyllin (**8**) and 3-hydroxyterphenyllin (**9**)

One liter of fermentation broth of MF6941 was extracted with MEK (1.3 l). The MEK layer was separated and the aqueous layer was washed twice with 250 ml MEK. The MEK extracts were pooled and concentrated to give 6.82 g of residue. A portion (6.1 g, 90%) of the residue was chromatographed on a silica-gel column (5×85 cm) and eluted with a gradient of hexane and 90% methyl *t*-butyl ether-CH_3CN (80:20 v/v) over 6 h at a flow rate of 10 ml/min, collecting fractions every 2.5 min. Fractions 133–135 and 139–146 were pooled and concentrated under reduced pressure to yield 18.3 (20 mg/l) and 270 mg (300 mg/l) terphyllin (**8**) and 3-hydroxyterphyllin (**9**), respectively, as colorless powders.

Fermentation of *Penicillium islandicum* for production of (+)-rugulosin (**10**)

The producing organism, MF6946, was isolated from a sewage sample collected in Poland using a conventional dilution plating method [38] and identified as *Penicillium islandicum* Sopp (Eurotiales anamorph, Ascomycetes) based on the typical brilliant-orange umbonate colonies formed on Czapek yeast extract agar and malt extract agar, producing consistently biverticillate penicilli [41]. Seed flasks were prepared from fresh slants of isolate MF6946 in PDA as described in [39]. Two-ml portions of the resulting culture were used to inoculate 250-ml unbaffled Erlenmeyer flasks containing 50 ml CYS80 medium [39], which were incubated at 25 °C on a rotatory shaker at 220 rpm for 21 days.

Extraction and isolation of (+)-rugulosin (**10**)

A 1.85-l fermentation broth of MF6946 was extracted with 1.85 l MEK and concentrated to dryness on a rotary evaporator. The residue of the MEK extract was dissolved in a 200 ml of a 1:1 mixture of methylene chloride-methanol, and chromatographed on a 2-l Sephadex LH20 column that was eluted with methanol. The HIV-1 integrase inhibitory activity eluted in fractions corresponding to 1,400–2,400 ml elution volume. The active fractions were combined and concentrated to dryness to furnish 2.7 g of an active pool enriched with rugulosin. A 200-mg portion of the active pool was chromatographed on a Zorbax RX C-8 (21×250 mm) column and eluted with a 60-min gradient of 20–80% aqueous CH_3CN + 0.1% TFA at a flow rate of 8 ml/min. The activity eluted in 46–48 min; the respective fractions were pooled and lyophilized to give 28 mg (378 mg/l) of pure rugulosin (**10**) as a yellow powder.

t_R 5.8 min (Zorbax RX C-8, 4.6×250 mm, 85% aqueous CH_3CN + 0.1% TFA, 1 ml/min), $[\alpha]^{25}_D = 582.5°$ (c, 0.4, methanol), HREIMS (*m*/*z*): 542.1225 (M^+, calculated for $C_{30}H_{22}O_{11}$: 542.1213).

Fermentation of an unidentified fungus (MF6074) for the production of compounds **11** and **12**

The producing organism, an unidentified fungus, was isolated from leaf litter collected in the Osa Peninsula, Costa Rica. For production of the compound, seed flasks were prepared from frozen vegetative mycelia of the isolate MF6074 as described [13]. Ten-ml aliquots of the seed culture were used to inoculate AD2 medium. The roller-bottle production vessels were incubated on a Wheaton roller machine at 22 °C and 70% relative humidity for 19 days.

Extraction and isolation of mixture of roselipins 2A (**11**) and 2B (**12**)

The solid fungal growth described above was extracted with 250 ml MEK per roller bottle. A 100-ml aliquot of the extract was separated and concentrated to dryness under reduced pressure on a

rotary evaporator to give 600 mg of a gum. This was subjected to gel-permeation chromatography on a 1-l Sephadex LH20 column in methanol. The activity eluting from an elution volume of 425–550 ml was dried to produce a gum (371.3 mg). This material was chromatographed on a reversed phase HPLC (Zorbax RX C-8, 21×250 mm) column and eluted with a linear gradient of 30–80% aqueous $CH_3CN + 0.1\%$ TFA at a flow rate of 10 ml/min. The activity eluted in fractions obtained at 47–48 min. These fractions were concentrated under reduced pressure and lyophilized to give 36.6 mg (366 mg/l) of chromatographically homogeneous, hygroscopic solid characterized as a mixture of roselipins 2A and 2B.

t_R 4.3 min (Zorbax RX C-8, 4.6×250 mm, 60% aqueous $CH_3CN + 0.1\%$ TFA, 1 ml/min), HRFABMS (*m/z*): 841.4880 ($[M + Na]^+$, calculated for $C_{42}H_{74}O_{15} + Na$: 841.4927)

Fermentation of *Neosartorya* sp. for the production of ophiobolins (**14**, **15**)

The producing organism, MF6942, was isolated from a sample of dung of Malabar squirrel, collected in Bondla, Goa (India), using the pasteurization method with ethanol as described by Bills and Polishook [7]. The fungus produced white- to cream-colored colonies on PDA and potato carrot agar [41], with cleistothecia 300–400 μm in diameter scattered throughout the colony surface, having a pseudoparenchymatous peridial layer, containing asci subglobose 13 μm in diameter, and bivalve ascospores 6.5 μm in diameter, with roughened walls and two equatorial crests. This morphology is consistent with the ascription of the strain to the Eurotiales [2], but the lack of an anamorph prevented a more accurate identification based on morphological characters. As an alternative approach to identification of the fungus, the sequence of the D1-D2 region of the 28S rRNA (539 bp) was obtained and compared with the GenBank database, following the method previously described [42]. The sequence of strain MF6942 (deposited in GenBank under accession number AY292369) showed more than 99% identity with the sequences of several *Neosartorya* species (Eurotiales, Ascomycetes) and their anamorphs. Although these data did not enable us to identify the strain at the species level, the similarity is sufficiently high to label the strain unequivocally as a species of genus *Neosartorya*. Two-ml portions of the seed culture, prepared from a fresh isolate of MF6942 in PDA [39], were used to inoculate F1 medium in 250-ml Erlenmeyer flasks [29]. Production flasks were incubated under static conditions at 25 °C and 50% relative humidity for 28 days.

Extraction and isolation of ophiobolin (**14**, **15**)

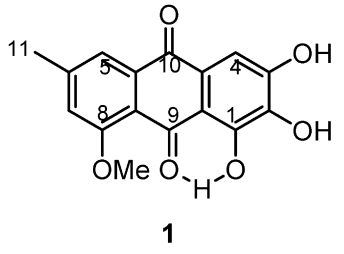
The fermentation broth (1.5 l) of MF6942 was extracted with 3 l MEK by shaking on a shaker for 30 min. The MEK layer was concentrated under reduced pressure and was chromatographed on a 2-l Sephadex LH20 column eluted with methanol. The active fractions eluted in a broad zone (1,000–1,300 ml). These fractions were pooled and concentrated to give 3.5 g of solid material, which was chromatographed on a silica-gel column and eluted with 10% acetone in hexane to produce 196 mg of an oily active cut. A 20-mg portion of this active cut was purified on a reversed phase HPLC column (Zorbax RX C-8, 21×250 mm). Elution with 65% aqueous $CH_3CN + 0.1\%$ TFA at a flow rate of 8 ml/min gave 1 mg epiophiobolin K (**14**), eluted in between 26 and 30 min, and 0.5 mg epiophiobolin C (**15**), eluted in between 31 and 35 min, as colorless gums.

Results and discussion

8-*O*-Methylanthrogallol (**1**)

This compound was produced by a strain of *Cylindrocarpon ianthothele* grown on CYS80 medium for 21 days. The fermentation broth was extracted with MEK and chromatographed on Sephadex LH20 in methanol followed by reversed phase HPLC to yield 8-*O*-methylanthrogallol (35 mg/l) as a yellow-orange powder.

High-resolution mass spectral analysis of **1** (Fig. 1) provided a molecular formula of $C_{16}H_{12}O_6$. The UV spectrum showed absorption maxima at 210, 280, 400 nm, which were similar to the spectrum of emodin. The molecular formula was corroborated by the ^{13}C NMR spectrum, which displayed 16 lines appearing in the downfield region of the spectrum except for an aromatic methoxy carbon at δ_C 57.2 and an aromatic methyl at δ_C 22.6. In addition, it showed a pair of signals appearing at δ_C 182.8 and 188.9 easily assigned to *para*-quinone carbonyls of a naphthoquinone. The presence of the methoxy and the methyl groups was confirmed by the presence of two three-proton singlets at δ_H 4.01 and 2.50, respectively, in the ^1H NMR spectrum recorded in acetone-d_6. The ^1H NMR spectrum revealed the presence of three aromatic singlets (δ_H 7.28, 7.37, and 7.66), an aromatic methyl singlet (δ_H 2.50) and one chelated phenolic singlet (δ_C 13.5). The comparison of the NMR spectrum of **1** with that of the corresponding spectrum of emodin indicated that a hydroxy group had replaced one of the aromatic protons of emodin in **1**. In addition, one of the hydroxy groups was methylated in **1**. The carbon-bearing protons were assigned by an HMQC experiment and the full structure of **1** was elucidated by an HMBC ($^nJ_{XH} = 7$ Hz) experiment. The HMBC correlations are summarized in Fig. 2. The methoxy protons (δ_H 4.01) produced HMBC correlation to C-8 (δ_C 162.4); the methyl protons (δ_H 2.50) afforded correlations to C-5 (δ_C 121.6), C-6 (δ_C 148.4) and C-7 (δ_C



1

Fig. 1 Structure of compound **1**

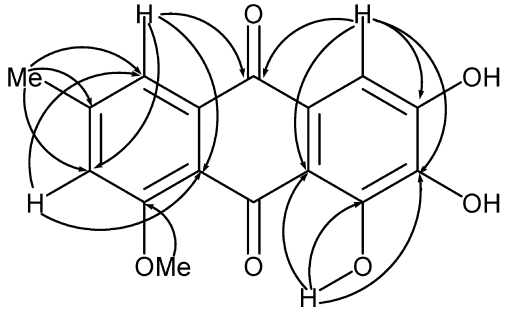


Fig. 2 HMBC correlations of **1** in acetone-d_6 ($^nJ_{XH} = 7$ Hz)

121.1); H-5 showed three-bond HMBC correlations to the carbonyl C-10 (δ_C 182.8), C-7 (δ_C 120.1), the methyl at C-11 (δ_C 22.6), and C-8a (δ_C 119.6); and H-7 exhibited three-bond HMBC correlations to C-5 (δ_C 121.6) and C-8a. These correlations established the methyl and methoxy substitutions in one of the aromatic rings and the trihydroxy substitution in the other aromatic ring. The most shielded of the aromatic protons, H-4 (δ_H 7.28), displayed an HMBC correlation to the same quinone carbonyl (C-10) that had shown HMBC correlation to H-5, thus establishing that H-4 and H-5 were flanking the quinone carbonyl C-10. In addition, H-4 showed three-bond HMBC correlations to C-2 (δ_C 139.8) and C-9a (δ_C 112.7) and two-bond correlation to C-3 (δ_C 151.7). Finally, the chelated hydroxy proton (δ_H 13.5) exhibited three HMBC correlations to C-1 (δ_C 152.8), C-2 (δ_C 139.8) and C-9a (δ_C 112.7) and unambiguously established the substitution pattern in the second aromatic ring and the structure as the new compound 8-*O*-methylanthrogallol (**1**) which is the 8-*O*-methyl derivative of anthrogallol (2-hydroxyemodin). Anthragallol was present in the insect extracts of *Eriococcus coriaceus*, *E. confusus*, *Nipaecoccus aurilanatus* (Maskell), and *Pseudococcus albizziae* (Maskell) [5].

Hispidin (**2**) and caffeic acid (**3**)

The producing fungus (MF6944) was grown in F1 medium. The culture broth was extracted with MEK and purified by two successive steps of reversed phase HPLC to afford hispidin (**2**) and caffeic acid (**3**) (Fig. 3). The structure of caffeic acid (**3**) was elucidated by comparison (HPLC, ^{1}H NMR, and IR) with an authentic sample. The structure of hispidin (**2**) was elucidated by 2D NMR spectral analysis, including HMBC experiments. The full assignment is listed in the Materials and methods section and is consistent with the reported values [1]. The H-3 was exchanged with deuterium when a ^{1}H NMR spectrum was recorded in methanol-d_4 due to the keto-enol tautomerization. This phenomenon was less evident in a spectrum recorded in acetone-d_6. Reaction of hispidin with diazomethane in a 1:1 mixture of methylene chloride and methanol exclusively produced trimethyl ether (**4**), which helped in the structure validation. Hispidin was first detected in *Polyporus hispidus* in 1961 [14] and the structure was confirmed by a total synthesis [15]. It has been subsequently found in various related fungal species, exemplified by *Inonotus hispidus* [3] and *Phellinus pomaceus* [25]. Hispidin inhibits protein kinase C (PKC) β-isoform ($IC_{50} = 2$ μM) and is preferentially cytotoxic to cancer cells [20]. It has been reported to show antioxidant activity by inhibiting lipid peroxidation in rat liver homogenates [24]. Inhibition of PKC by hispidin has been associated with correction of hyperpermeability of albumin in isolated diabetic venules [67].

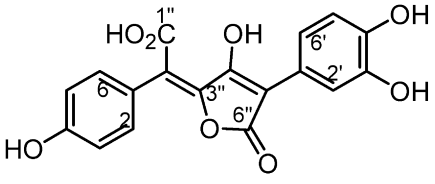
Xerocomic acid (**5**)

Xerocomic acid (**5**) (Fig. 4) was produced by a strain of *Xeromphalina junipericola* grown on BRFT medium. An MEK extract of the fermentation broth was chromatographed on Sephadex LH20 followed by reversed phase HPLC to yield xerocomic acid. HREIMS produced a molecular formula of $C_{18}H_{12}O_8$. The search of this formula in the Chapman Hall database produced many hits, including xerocomic acid. The structure was confirmed by 2D NMR spectral analysis [56]. Full ^{1}H and ^{13}C NMR assignment of xerocomic acid (**5**) is listed in Materials and methods.

Xerocomic acid was first isolated from *Xerocomus chrysenteron* in 1968 by Steglich et al. [57] and was subsequently detected in various basidiomycetous species belonging to the order Boletales [6, 18, 63]. We isolated the compound from *X. junipericola*, a species from Spain that was described earlier [30]. Our data constitute the first report of the isolation of xerocomic acid from a member of the Agaricales. No other biological activity of xerocomic acid has been reported.

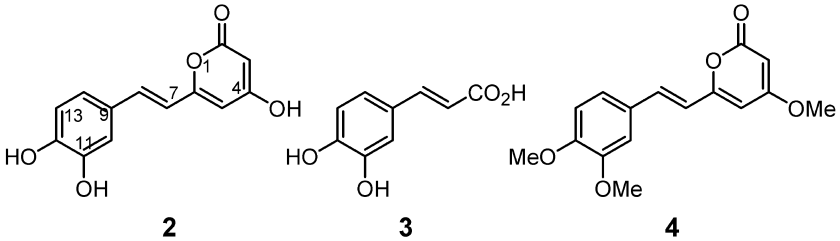
Deoxyfunicone (**6**)

This compound was produced by a strain of *Penicillium* sp. grown on AD2 medium. The medium was extracted with MEK and purified by a bioactivity-guided isola-



5

Fig. 4 Structure of xerocomic acid (**5**)



2 **3** **4**

Fig. 3 Structures of hispidin (**2**), caffeic acid (**3**), and trimethyl ether (**4**)

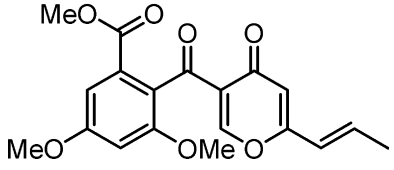
tion employing Sephadex LH20, reversed phase HPLC, and silica-gel TLC to yield deoxyfunicone (Fig. 5). HREIMS gave a molecular formula of $C_{19}H_{18}O_7$ and led to the identification of deoxyfunicone (**6**) by comparison with the published UV, IR, MS, and NMR spectral data [44].

Altenusin (**7**)

This compound was produced by a strain of *Talaromyces flavus* grown on CYS80 medium. It was extracted with MEK and purified by gel-permeation chromatography on Sephadex LH20 to yield altenusin (**7**) (Fig. 6) as the only HIV-1-integrase-active component. It was identified by comparison of the UV, MS, and ^{1}H NMR spectral data with corresponding published data [10]. Complete and unambiguous ^{1}H and ^{13}C NMR assignments, based on the HMBC experiment, are listed in Materials and methods. Altenusin was originally isolated from *Alternaria tenuis* [43] and subsequently isolated from various organisms, including *Penicillium* sp. [64]. It has been reported as a weak inhibitor of myosin light chain kinase ($IC_{50} = 340$ μM) [31], and sphingomyelinase ($IC_{50} = 28$ μM) [64].

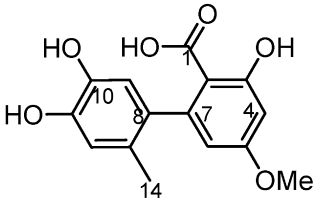
Terphenyllin (**8**) and hydroxyterphenyllin (**9**)

These compounds were produced by a strain of *Aspergillus candidus* grown on MV8 medium. Silica-gel chromatography of a MEK extract led to the isolation of terphenyllin (**8**) and hydroxyterphenyllin (**9**), respectively (Fig. 7). These compounds were characterized by comparison with the reported ^{1}H and ^{13}C NMR spectra [28].



6

Fig. 5 Structure of deoxyfunicone (**6**)



7

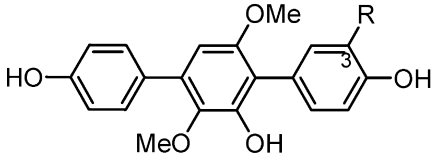
Fig. 6 Structure of altenusin (**7**)

(+)-Rugulosin (**10**)

Rugulosin was isolated from a strain of *Penicillium islandicum* grown on CYS80 medium. Extraction of the fermentation broth with MEK was followed by purification using gel filtration on Sephadex LH20 and reversed phase HPLC. HREIMS produced a molecular formula of $C_{30}H_{22}O_{10}$, which was consistent with the formula of rugulosin as revealed by the Chapman Hall database. The identity of the isolated compound was confirmed as (+)-rugulosin (**10**) (Fig. 8) by comparison of the observed and reported spectral (UV, MS, and ^{1}H and ^{13}C NMR) data and specific rotation [10]. Both (+)- and (−)-rugulosin have been reported from various species of *Penicillium* sp. Surprisingly the enantiomer (−)-rugulosin was reported from *P. islandicum* [10], indicating that the same culture is capable of producing both enantiomers.

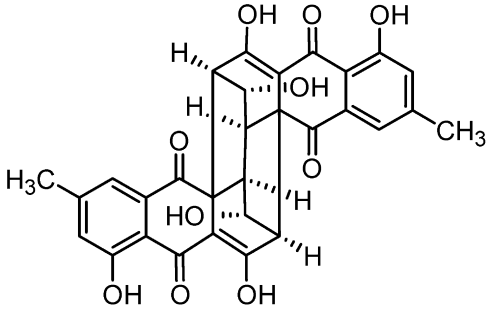
Roselipin 2A (**11**) and roselipin 2B (**12**)

These compounds were isolated from an unidentified fungus that was grown on an AD2 medium. The medium was extracted with MEK. Chromatography on Sephadex LH20 followed by reversed phase HPLC afforded a 1:1 mixture of isomers **11** and **12**. Although we isolated and structurally elucidated these compounds by extensive 2D NMR in 1994, the structure was first reported in 1999 by Omura and coworkers [59] of Kitasato Institute and Kohno et. al. [27] of Tanabe



8 (R = H)
9 (R = OH)

Fig. 7 Structures of terphenyllin (**8**) and hydroxyterphenyllin (**9**)



10

Fig. 8 Structure of rugulosin (**10**)

Seiyaku Co., isolated from *Gliocladium roseum* and *catenulatum,* respectively. The Kitasato group was able to separate them by a long HPLC run and named these compounds roselipins 2A (**11**) and 2B (**12**) (Fig. 9). D-Mannose and D-arabinitol were conclusively identified but the stereochemistry of the polyketide chain was not reported. The ^{1}H and ^{13}C NMR spectral data recorded in CD_3OD of the compound we had isolated were identical to the corresponding spectra of the mixture of roselipins 2A and 2B. In fact, the NMR spectra of roselipins 2A and 2B were identical except for the carbon signals originating from the arabinitol residue and C-1 to C-3 of the polyketide unit due to differential esterification of D-arabinitol. The Tanabe Seiyaku group reported the isolation of only one of the two isomers (**11**) and named it TMC-151F. In addition to D-arabinitol and D-mannose, they determined the stereochemistry of the polyketide chain by X-ray crystallography of the base hydrolytic product **13** [27]. In order to correlate roselipin 2A with TMC-151F, we recorded the ^{1}H and ^{13}C NMR spectra of our isolate in DMSO-d_6, which compared very well ($\Delta\delta_C \pm 0.1$ ppm) with the corresponding spectra reported for TMC-151F. In addition, analogous [27] alkaline hydrolysis of our isolate mixture **11** and **12** gave product **13**, which exhibited identical ^{1}H and ^{13}C spectral data to the hydrolysis product of TMC-151F. Despite the differences of optical activity of roselipin 2A and TMC-151F, these data would indicate that the structures of roselipin 2A and TMC-151F (11) are identical.

TMC-151F was reported to show cytotoxicity against several tumor cell lines. Roselipins [5, 60, 61] were shown to be inhibitors of diacylglycerol acyltransferase (DGAT) and also as anti *Aspergillus* agents.

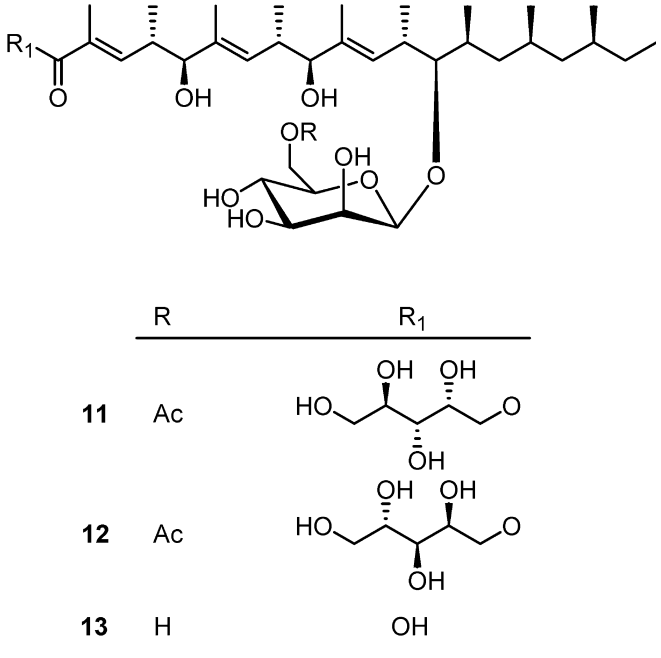


Fig. 9 Structures of compounds **11**, **12**, and **13**

Ophiobolins

Epiophiobolin K (**14**) and epiophiobolin C (**15**) were produced by a strain of a *Neosartorya* sp. grown on F1 medium extracted with MEK. Purification of these products by three successive steps comprising a Sephadex LH20 column, silica gel chromatography, and preparative reversed phase HPLC afforded epiophiobolin K and epiophiobolin C. The structures of these compounds were elucidated by comparison of the ^{1}H NMR spectral data and co-injection on HPLC data with respective authentic samples. The occurrence and biological activities of ophiobolins were recently reviewed [4].

Nalanthalide (**21**) and coprophilin (**22**)

Nalanthalide (**21**) and coprophilin (**22**) were originally isolated as a blocker of the voltage-gated potassium channel [19] and an anticoccidial agent [36], respectively. They were selected to test against HIV-1 integrase based on their decalin structural similarity to the known HIV integrase inhibitors integric acid and equisetin.

Biological activity

All compounds were evaluated for their ability to inhibit HIV-1 integrase in coupled (simultaneously measuring 3′-end processing and strand-transfer reactions) and strand-transfer assays. The data are summarized in Table 1.

The inhibitors described here were grouped in two major classes. (1) Those possessing more than one phenolic group are represented by compounds **1**–**10,** and (2)

Table 1 HIV-1 integrase inhibitory activities of natural products (1–22). *NA (100)* Not active at 100 μM, *NT* not tested

Compound	Coupled assay (IC_{50}, μM)	Strand transfer assay (IC_{50}, μM)
1	6	22
2	2	24
3	2.8	24
4	NA (100)	NA (100)
5	1.1	4.4
6	11	>140
7	19	25
8	17.7	47.7
9	2.8	12.1
10	19	25
11 + 12	8.5	NT
14	19	>120
15	33	>120
16	48	>125
17	21.5	125
18	6.7	33
19	30	>120
20	23	>120
21	10	25
22	10	25

those belonging to either terpenoid or polyketide classes and not containing a phenolic group are represented by compounds **11**–**22**.

Of the first group, the compounds with a catechol residue, **1**, **2**, **3**, **5**, and **9**, were the most active in the coupled assay and had IC_{50} values of 6, 2, 2.8, 1.1 and 2.8 μM, respectively. However, the strand-transfer activity of these compounds was 4- to 12-fold (Table 1) lower than the coupled activity, indicating that these compounds are most likely cleavage inhibitors. The remaining compounds (**6**–**8**, and **10**) of the first group were less active in both assays, with IC_{50} values of 11–19 μM in the coupled assay and 12.1–>140 μM in the strand-transfer assay (Table 1). Both assay activities were completely abolished when the phenolic groups of hispidin were capped as a methyl ether (**4**), indicating the critical role played by the acidic phenolic groups in the integrase inhibitory activity, a known phenomenon of this enzyme. A derivative of caffeic acid, caffeic acid phenethyl ester (CAPE), was reported to inhibit the strand-transfer reaction with $IC_{50} = 18.9$ μM and was a surprisingly poor inhibitor of the cleavage reaction ($IC_{50} = 220$ μM) [16, 17].

Of the second group of compounds, the 1:1 mixture of **11** and **12**, **18**, **21**, and **22** exhibited better activity in the coupled assay compared to compounds **14**–**17**, **19**, and **20**, with IC_{50} values of 8.7, 6.7, 10, and 10 μM, respectively (Table 1). The equisetin-type decalin-containing compounds nalanthalide (**21**) and coprophilin (**22**) were equipotent and were 2.5-fold better inhibitors of the coupled reaction than of the strand transfer reaction.

Of the ophiobolins, epiophiobolins K (**14**) and C (**15**) exhibited HIV-1-integrase inhibitory activities with IC_{50} values of 29 and 33 μM, respectively, in the coupled assay but both were inactive in the strand-transfer assay ($IC_{50} = >120$ μM). To evaluate the SAR (structure activity relationship), several previously reported [45, 62] ophiobolins were evaluated by these assays. These included ophiobolins A (**16**), B (**17**), C (**18**), H (**19**), and K (**20**), which exhibited IC_{50} values of 48, 21.5, 6.7, 30, and 23 μM, respectively, against the coupled assay and >125, 125, 33, >120, and >120 μM, respectively, against the strand-transfer assay. Amongst these, the *cis*-fused A/B ring ophiobolin C was the most active.

Conclusions

In summary, we have reported the structure and HIV-1-integrase inhibitory activity of 21 diverse natural products broadly represented by two groups: (1) containing more than one phenolic group, and (2) terpenoids (e.g., ophiobolins, **14**–**20**), and polyketides (**11**, **12**, **21**, and **22**). As observed earlier, the catechol residue seems to be important for the potency of the phenolic compounds regardless of the structural type. HIV-1 integrase appears to have a recognition site for the decalin structural unit, exemplified by the similar activities of equisetin, phomasetin, nalanthalide, and coprophilin. Structural diversity of these compounds indicates that many of these inhibitors are not active-site inhibitors but rather allosteric inhibitors.

Acknowledgements The authors are thankful to Ms. J. Patel, M. Arocho, and D. Valentino for their invaluable assistance in the initial extraction and supply of screening samples.

References

1. Adam, W, Saha-Moller, C R, Veit, M, Welke, B (1994) A convenient synthesis of hispidin from piperonal. Synthesis1133–1134
2. Ainsworth GC, Sparrow FK, Sussman A (eds) (1973) The fungi an advanced treatise. Academic, London
3. Ali NA, Jansen R, Pilgrim H, Liberra K, Lindequist U (1996) Hispolon, a yellow pigment from *Inonotus hispidus*. Phytochemistry 41:927–929
4. Au TK, Chick WSH, Leung PC (2000) The biology of ophiobolins. Life Sci 67:733–742
5. Banks HJ, Cameron DW, Raverty WD (1976) Chemistry of the Coccoidea. II. Condensed polycyclic pigments from two Australian Pseudococcids (Hemiptera). Aust J Chem 29:1509–1521
6. Besl H, Bresinsky A (1977) Presence and systematic evaluation of pigments in higher fungi 2. Z Pilzkd 43:311–322
7. Bills GF, Polishook J (1993) Selective isolation of fungi from dung of *Odocoileus hemionus* (mule deer). Nova Hedwigia 57:195–206
8. Bills GF, Polishook J (1994) Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. Mycologia 86:187–198
9. Booth C (1966) The genus *Cylindrocarpon*, vol 104, CMI Kew, Surrey, UK
10. Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic, New York
11. Craigie R (2001) HIV integrase, a brief overview from chemistry to therapeutics. J Biol Chem 276:23213–23216
12. De Clercq E (2002) New anti-HIV agents and targets. Med Res Rev 22:531–565
13. Dombrowski AW, Jenkins R, Raghoobar S, Bills GF, Polishook JD, Peláez F, Burgess B, Zhao A, Huang L, Zhang Y, Goetz M (1999) Production of a family of kinase-inhibiting lactones from fungal fermentations. J Antibiot 52:1077–1085
14. Edwards RL, Lewis DG, Wilson DV (1961) Constituents of the higher fungi I Hispidin, a new 4-hydroxy-6-styryl-2-pyrone from *Polyporus hispidus*. J Chem Soc 4995–5002
15. Edwards RL, Wilson DV (1961) Constituents of the higher fungi II Synthesis of hispidin J Chem Soc5003–5004
16. Fesen M, Kohn K, Leteurtre F, Pommier Y (1993) Inhibitors of human immunodeficiency virus integrase. Proc Natl Acad Sci USA 90:2399–2403
17. Fesen MR, Pommier Y, Leteurtre F, Hiroguchi S, Yung J, Kohn KW (1994) Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds Biochem Pharmacol 48:595–608
18. Gill M, Steglich W (1987) Pigments of fungi (Macromycetes) In: Hertz W, Grisebach H, Kirby GW, Tamm C (eds) Progress in the chemistry of organic natural products. Springer, Vienna, pp 1–287
19. Goetz MA, Zink DL, Dezeny G, Dombrowski A, Polishook JD, Felix JP, Slaughter RS, Singh SB (2001) Diterpenoid pyrones, novel blockers of the voltage-gated potassium channel Kv13 from fungal fermentations. Tetrahedron Lett 42:1255–1257
20. Gonindard C, Bergonzi C, Denier C, Sergheraert C, Klaebe A, Chavant L, Hollande E (1997) Synthetic hispidin, a PKC inhibitor, is more cytotoxic toward cancer cells than normal cells in vitro. Cell Biol Toxicol 13:141–153

21. Hazuda D, Blau CU, Felock P, Hastings J, Lineberger D, Pramanik B, Wolfe A, Buchman F, Farnet C, Goetz M, Williams M, Silverman KC, Lingham R, Singh S (1999) Isolation and characterization of a novel class of human immunodeficiency virus integrase inhibitors from natural product screening. Antivir Chem Chemother 10:63–70
22. Hazuda, DJ, Felock P, Witmer M, Wolfe A, Stillmock K, Grobler JA, Espeseth A, Gabryelski L, Schleif W, Blau C, Miller MD (2000) Inhibitors of strand transfer that prevent integration and inhibit hiv-1 replication in cells. Science 287:646–650
23. Jayasuriya H, Guan Z, Polishook JD, Dombrowski AW, Felock PJ, Hazuda DJ, Singh SB (2003) Isolation, structure and HIV-1 integrase-inhibitory activity of cytosporic acid, a fungal metabolite produced by a *Cytospora* sp. J Nat Prod 66:551–553
24. Khushbaktova ZA, Yusupovam SM, Badalyants KL, Syrov VN (1996) Isolation and antioxidant activity of hispidin from walnut fungus. Khim Prir Soedin34–37
25. Klarr M, Steglich W (1977) Fungus pigments, XXVII Isolation of hispidin and 3, 14′-bihispidinyl from *Phellinus pomaceus* (Poriales). Chem Ber 110:1058–1062
26. Klich MA (2002) Identification of common *Aspergillus* species. CBS, Baarn, The Netherlands
27. Kohno J, Nishio M, Sakurai M, Kawano K, Hajime H, Noriaki N, Kishi N, Yamashita T, Okuda T, Komatsubara S (1999) Isolation and structure determination of TMC-151 s: Novel polyketide antibiotics from *Gliocladium catenulatum* Gilma and Abbott TC 1280. Tetrahedron 55:7771–7786
28. Kurobane I, Vining LC, McInnes AG, Smith DG (1979) 3-Hydroxyterphenyllin, a new metabolite of *Aspergillus candidus*. J Antibiot 32:559
29. Mandala SM, Thornton RA, Frommer BR, Curotto JE, Rozdilsky W, Kurtz MB, Giacobbe RA, Bills GF, Cabello A, Martín I, Peláez F, Harris GH (1995) The discovery of australifungin, a novel inhibitor of sphinganine N-acyltransferase from *Sporormiella australis*. J Antibiot 48:349–356
30. Moreno G, Heykoop M (1996) *Xeromphalina junipericola* sp. nov. (Tricholomataceae, Agaricales) from Spain. Z Mykol 62:37–41
31. Nakanishi S, Toki S, Saitoh Y, Tsukuda E, Kawahara K, Ando K, Matsuda Y (1995) Isolation of myosin light chain kinase inhibitors from microorganisms: dehydroaltenusin, altenusin, atrovenetinone, cyclooctasulfur. Biosci Biotech Biochem 59:1333–1335
32. Neamati N (2001) Structure-based HIV-1 integrase-inhibitor design: a future perspective. Exp Opin Invest Drugs 10:281–296
33. Neamati N (2002) Patented small molecule inhibitors of HIV-1 integrase: a 10-year saga. Exp Opin Ther Patents 12:709–724
34. Neamati N (2003) Dipyrimidine-based inhibitors of HIV-1 integrase. Exp Opin Invest Drugs 12:289–292
35. Omura S, Tomoda H, Ohyama Y, Tabata N, Abe T, Namikoshi M (1999) Roselipins, novel fungal metabolites having a highly methylated fatty acid modified with a mannose and an arabinitol. J Antibiot 52:586–589
36. Ondeyka JG, Giacobbe RA, Bills GF, Cuadrillero C, Schmatz D, Goetz MA, Zink DL, Singh SB (1998) Coprophilin: an anticoccidial agent produced by a dung inhabiting fungus. Bioorg Med Chem Lett 8:3439–3442
37. Pais GCG, Burke JTR (2002) Novel aryl diketo-containing inhibitors of HIV-1 integrase. Drugs of Future 27:1101–1111
38. Parkinson D (1994) Filamentous fungi. In: Weaver RW, Angle S, Bottomley P, Bezdieck D, Smith S (eds) Methods of soil analysis. Part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, Wisconsin, USA
39. Peláez F, Collado J, Arenal F, Basilio A, Cabello A, Díez MT, García JB, González del Val A, González V, Gorrochategui J, Hernández P, Martín I, Platas G, Vicente F (1998) Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycol Res 102:755–761
40. Peláez F, Cabello A, Platas G, Díez MT, González del Val A, Basilio A, Martín I, Vicente F, Bills GF, Giacobbe RA, Onishi JC, Meinz M, Abruzzo G, Flattery A, Kong L, Kurtz MB (2000) The discovery of enfumafungin, a novel antifungal compound produced by an endophytic *Hormonema* species Biological activity and taxonomy of the producing organism. Syst Appl Microbiol 23:333–343
41. Pitt JI (2000) A laboratory guide to common *Penicillium* species, 3rd edn. CSIRO Division of Food Processing, North Ryde, NSW
42. Polishook JD, Peláez F, Platas G, Asensio FJ, Bills GF (2000) Observations on aspergilli in Santa Rosa National Park, Costa Rica. Fungal Diversity 4:81–100
43. Rosett T, Sankhala RH, Stickings CE, Taylor MEU, Thomas R (1957) Studies in the biochemistry of micro-organisms 103 Metabolites of *Alternaria tenuis* Auct: Culture filtrate products. Biochem J 67:390–400
44. Sassa T, Nukina M, Suzuki Y (1991) Deoxyfunicone, a new gamma-pyrone metabolite form a resorcylide-producing fungus (*Penicillium* sp). Agric Biol Chem 55:2415–2416
45. Singh SB, Smith JL, Sabnis GS, Dombrowski AW, Schaeffer JM, Goetz MA, Bills GF (1991) Structure and conformation of ophiobolin K and 6- epiophiobolin K from *Aspergillus ustus* as a nematocidal agent. Tetrahedron 47:6931–6938
46. Singh SB, Zink DL, Goetz MA, Dombrowski AW, Polishook JD, Hazuda DJ (1998) Equisetin and a novel opposite stereochemical homolog phomasetin, two fungal metabolites as inhibitors of HIV-1 integrase. Tetrahedron Lett 39:2243–2246
47. Singh SB, Zink D, Polishook J, Valentino D, Shafiee A, Silverman K, Felock P, Teran A, Vilella D, Hazuda DJ, Lingham RB (1999) Structure and absolute stereochemistry of HIV-1 integrase-inhibitor integric acid, a novel eremophilane sesquiterpenoid produced by a *Xylaria* sp. Tetrahedron Lett 40:8775–8779
48. Singh SB, Felock P, Hazuda DJ (2000) Chemical and enzymatic modifications of integric acid and HIV-1 integrase-inhibitory activity. Bioorg Med Chem Lett 10:235–237
49. Singh SB, Jayasuriya H, Salituro GM, Zink DL, Shafiee A, Heimbach B, Silverman KC, Lingham RB, Genilloud O, Teran A, Vilella D, Felock P, Hazuda D (2001) The complestatins as HIV-1 integrase-inhibitor Efficient isolation, structure elucidation and inhibitory activities of isocomplestatin, chloropeptin I, new complestatins, A and B, acid-hydrolysis products of chloropeptin I. J Nat Prod 64:874–882
50. Singh SB, Zink DL, Bills GF, Pelaez F, Teran A, Collado J, Silverman K C, Lingham RB, Felock P, Hazuda D J (2002) Discovery, structure and HIV-1 integrase-inhibitory activities of integracins, novel dimeric alkyl aromatics from *Cytonaema* sp. Tetrahedron Lett 43:1617–1620
51. Singh SB, Zink DL, Quamina DL, Pelaez F, Teran A, Felock PJ, Hazuda DJ (2002) Integrastatins: structure and HIV-1 integrase-inhibitory activities of two novel racemic tetracyclic aromatic heterocycles produced by two fungal species. Tetrahedron Lett 43:2351–2354
52. Singh SB, Zink DL, Heimbach B, Genilloud O, Teran A, Silverman KC, Lingham RB, Felock P, Hazuda DJ (2002) Structure, stereochemistry and biological activity of integramycin, a novel hexacyclic natural product produced by actinoplanes sp. that inhibits hiv-1 integrase. Organic Lett 4:1123–1126
53. Singh SB, Herath K, Guan Z, Zink DL, Dombrowski AW, Polishook JD, Silverman KC, Lingham RB, Felock PJ, Hazuda DJ (2002) Integramide A and B, two novel non-ribosomal linear peptides containing nine Cα-methyl amino acids produced by fungal fermentations that are inhibitors of hiv-1 integrase. Org Lett 4:1431–1434, 3337
54. Singh SB, Zink DL, Dombrowski AW, Polishook JD, Ondeyka JG, Hirshfield J, Felock P, Hazuda DJ (2003) Integracides: tetracyclic triterpenoid inhibitors of hiv-1 integrase produced by *Fusarium* sp. Bioorg Med Chem 11:1577–1582

55. Singh SB, Zink DL, Bills GF, Teran A, Silverman KC, Lingham RB, Felock P, Hazuda DJ (2003) Four novel bis- (naphtho-γ-pyrones) isolated from *Fusarium* species as inhibitors of HIV-1 integrase. Bioorg Med Chem Lett 13:713–717
56. Steglich W, Besl H, Zipfel K (1974) Pigments of fungi XIX Structural determination of pulvinic acids by NMR spectroscopy. Z Naturforsch, Teil B 29:96–98
57. Steglich W, Furtner W, Prox A (1968) New pulvinic acid derivatives from *Xerocomus chrysenteron* and occurrence of anthraquinone pigments in Boletaceae. Z Naturforsch, Teil B 23:1044–1050
58. Stolk AC, Samson RA (1972) The genus *Talaromyces*, study mycology, vol 2. CBS, Baarn, The Netherlands
59. Tabata N, Ohyama Y, Tomoda H, Abe T, Namikoshi M, Omura S (1999) Structure Elucidation of Roselipins, Inhibitors of diacylglycerol acyltransferase produced by *Gliocladium roseum* KF-1040. J Antibiot 52:815–826
60. Tomoda H, Ohyama Y, Abe T, Tabata N, Namikoshi M, Yamaguchi Y, Masuma R, Omura S (1999) Roselipins, Inhibitors of diacylglycerol acyltransferase produced by *Gliocladium roseum* KF-1040. J Antibiot 52:689–694
61. Tomoda H, Tabata N, Ohyama Y, Omura S (2003) Core structure in roselipins essential for eliciting inhibitory activity against diacylglycerol acyltransferase. J Antibiot 56:24–29
62. Tsipouras A, Adefarati AA, Tkacz JS, Frazier EG, Rohrer SP, Birzin E, Rosegay A, Zink DL, Goetz MA, Singh SB, Schaeffer JM (1996) Ophiobolin m and analogues, noncompetitive inhibitors of ivermectin binding with nematocidal activity. Bioorg Med Chem 4:531–536
63. Turner WB, Aldridge DC (1983) *F*ungal Metabolites II. Academic, London
64. Uchida R, Tomoda H, Dong Y, Omura S (1999) Altenusin, a specific neutral sphingomyelinase inhibitor, produced by *Penicillium* sp FO-7436. J Antibiot 52:572–574
65. Wai JS, Egbertson MS, Payne LS, Fisher TE, Embrey MW, Tran LO, Melamed JY, Langford HM, Guare JJP, Zhuang L, Grey VE, Vacca JP, Holloway MK, Naylor-Olsen AM, Hazuda DJ, Felock PJ, Wolfe AL, Stillmock KA, Schleif WA, Gabryelski LJ, Young SD (2000) 4-Aryl-2,4-dioxybutanoic acid inhibitors of HIV-1 integrase and viral replication in cells. J Med Chem 43:4923–4926
66. Worrall JJ (1992) Media for selective isolation of Hymenomycetes. Mycologia 83:296–302
67. Yuan SY, Ustinova EE, Wu MH, Tinsley JH, Xu W, Korompai FL, Taulman AC (2000) Protein kinase C activation contributes to microvascular barriier dysfunction in the heart at early stages of diabetes. Circ Res 87:412–417
68. Zhuang L, Wai JS, Embrey MW, Fisher TE, Egbertson MS, Payne LS, Guare JJP, Vacca JP, Hazuda DJ, Felock PJ, Wolfe AL, Stillmock KA, Witmer MV, Moyer G, Schleif WA, Gabryelski LJ, Leonard YM, Lynch JJ, Michelson SR, Young SD (2003) Design and synthesis of 8-hydroxy-[1,6]naphthyridines as novel inhibitors of HIV-1 integrase in vitro and in infected cells. J Med Chem 46:453–456