Drugs of the Future 2005, 30(3): 277-299

CCC: 0377-8282/2005
DOI: 10.1358/dof.2005.030.03.885746

Review Article

Discovery of natural product inhibitors of HIV-1 integrase at Merck

Sheo B. Singh[1], Fernando Pelaez[2], Daria J. Hazuda[3], Russell B. Lingham[1]

[1]Merck Research Laboratories, P.O. Box 2000, Rahway, NJ 07065, USA; [2]CIBE, Merck Sharp & Dohme de España, S.A., Josefa Valcárcel 38, 28027 Madrid, Spain; [3]Merck Research Laboratories, West Point, PA 19486, USA.
Correspondence: e-mail: sheo_singh@merck.com

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Abstract

HIV is the causative agent of AIDS, which is currently clinically controlled by drugs that inhibit two of the three enzymes of the virus, namely reverse transcriptase and protease. The development of drugs targeting the third enzyme, integrase, has been elusive. HIV-1 integrase catalyzes the integration of viral DNA into host DNA using three different steps: the assembly of proviral DNA onto integrase, endonucleolytic cleavage of the proviral DNA and strand transfer of the proviral DNA into the host cell DNA. We conducted a screening effort focused on natural product extracts for the discovery of inhibitors of this enzyme and the results are reviewed in this article. The evaluation of over 200,000 extracts originating from more than 50,000 microbial strains equally represented by fungi and actinomycetes led to the discovery of 24 novel classes of chemically diverse natural product inhibitors of HIV-1 integrase, representing a wide variety of classes with molecular weights ranging from 180-1,663 Da and HIV-1 integrase-inhibitory activity IC_{50} values ranging from 50 nM to > 100 μM. Fungal strains were extremely productive and provided all but two classes of inhibitors. A β-hydroxyketo group was present in many of the compounds identified. If examined carefully, this feature could lead to further development of other compounds if additional 3-dimensional inhibitor-bound enzyme structural information becomes available.

Introduction

The causative agent of AIDS, namely, HIV-1, was identified almost 20 years ago (1-3). Since then, there has been a concerted global effort by academic and pharmaceutical organizations to first understand the pathophysiology of the disease and then identify chemical and biological therapeutics to halt the spread of the disease. There is a rich and diverse literature describing the structure and life cycle of the virus, which has been reviewed by others (4). A salient feature of HIV-1 virus replication is its high-affinity binding to CD4 on T-cells via gp120, a glycoprotein present on the viral envelope. Fusion of viral and host membranes occurs through another viral glycoprotein, gp41, which results in the exposure and internalization of viral RNA into the host cell. The viral reverse transcriptase enzyme reverse transcribes viral RNA into viral DNA, which migrates into the nucleus and is integrated into the host genome. This latter activity is performed by the viral enzyme integrase and results in the viral DNA being permanently incorporated into the host cellular DNA (Fig. 1). Replication of the virus generates regulatory (Tat, Rev and Nef) and accessory proteins (vif, vpr and vpu), along with enzymes (reverse transcriptase, integrase and protease) and genomic RNA, which are encapsulated by the host cell membrane. The maturation and release of new virus particles occur upon processing of the gag-pol protein by HIV-1 protease.

The complexity of the HIV-1 virus is belied by the paucity of enzymes or proteins that can be targeted to develop effective clinical therapeutics. Currently, there are 7 drugs that target HIV-1 protease (saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, lopinavir and atazanavir), 10 drugs that target HIV-1 reverse transcriptase (the nucleoside reverse transcriptase inhibitors [NRTIs] zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and tenofovir, and the non-nucleoside reverse transcriptase inhibitors [NNRTIs] nevirapine, delavirdine and efavirenz) and 1 fusion inhibitor (enfuvirtide [T-20]), which targets gp41. In addition, a significant effort is under way to develop vaccines against HIV-1, and several vaccine candidates are proceeding through

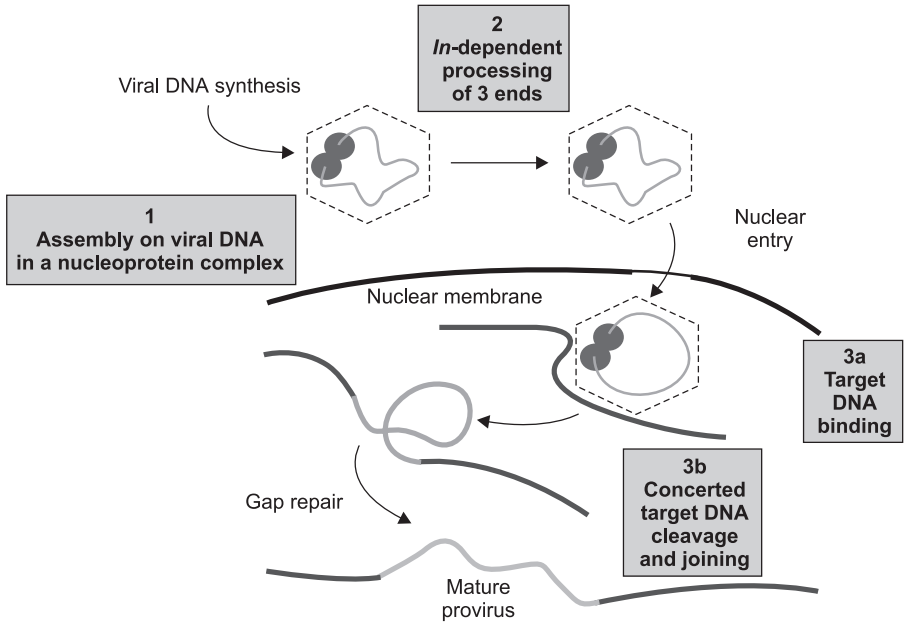


Fig. 1. Schematic representation of HIV-1 integrase-catalyzed functions.

the drug development process to determine their efficacy.

One of the major issues regarding effective treatment and management of the disease is the relentless development of resistance exhibited by the virus to new chemical therapeutics. The current clinically accepted paradigm to treat and manage HIV-1 infection is the use of combination therapy, or HAART (highly active antiretroviral therapy). It appears that the development of viral resistance to new drugs is inversely proportional to the time it takes to develop new therapeutics. The reason for this is the poor accuracy of viral replication, which generates a large number of viral variants that are insensitive to the drug being used. Strains of virus that appear to be resistant to most of the drugs currently available, including the recently introduced enfuvirtide, have emerged in the clinic (5). Thus, there is an urgent need to develop therapeutics that target drug-resistant strains of the virus, as well as to identify new targets within the virus, if the progression and spread of the disease are going to be impacted in any meaningful manner.

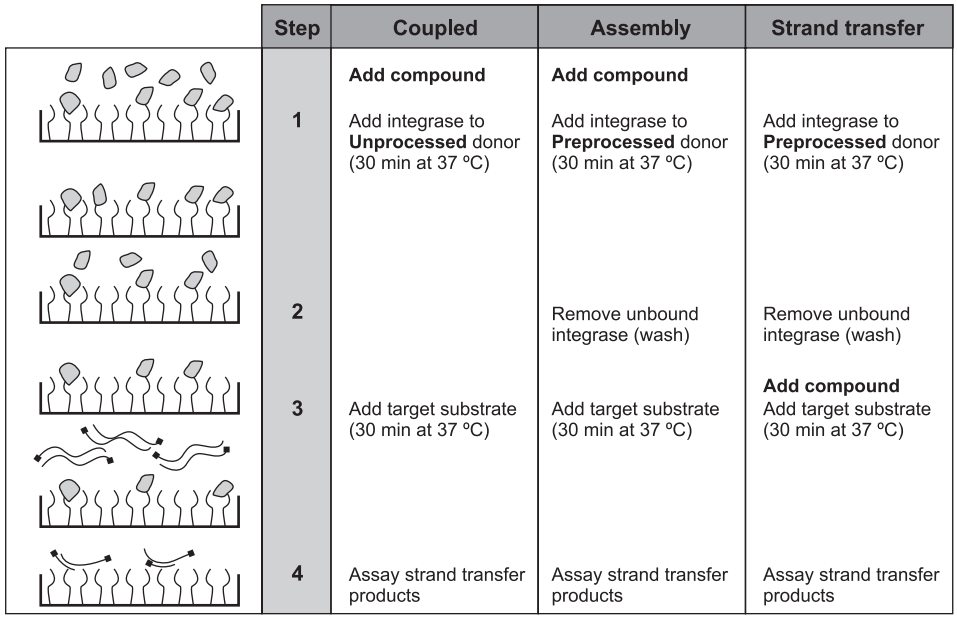
Of the three enzymes that comprise the HIV-1 virus, there are currently no commercially available drugs directed against HIV-1 integrase. HIV-1 integrase represents a valid target for the development of specific anti-HIV-1 agents. To this end, Merck & Co. has had a long-standing research effort into developing agents targeting all the HIV-1 enzymatic activities. These efforts have resulted in the commercialization of indinavir and efavirenz (developed by the former DuPont Merck and marketed by Bristol-Myers Squibb).

HIV-1 integrase is a 31-kDa protein that is required for integration of the viral DNA into the host genome. This enzyme is encoded by the 3'-end of the viral polymerase gene, and is processed from a larger precursor to yield active enzyme. Catalytically, HIV-1 integrase first interacts with long terminal repeats (LTRs) found on linear viral DNA; it then assembles the viral DNA in a nucleoprotein complex. The intrinsic endonuclease activity associated with HIV-1 integrase removes 2 bases from the 3'-hydroxyl termini of both linear strands of viral DNA, generating 2 recessed ends which serve as donor substrates for the strand transfer reaction. Entry of the processed viral DNA into the nucleus of the host cell then occurs. Integrase also nonspecifically creates staggered nicks in host DNA and transfers each recessed viral 3'-end onto the 5'-ends of the nicked host substrate (Fig. 1). HIV-1 integrase is the only enzyme known to perform these reactions.

Integration is essential for viral replication and is therefore a viable drug target. In order to identify inhibitors that could be developed as leads and drugs, a number of assays, including high-throughput screening assays, were developed and used to screen compound and natural product extract collections. Natural products are historically known to be rich sources of structurally diverse enzyme inhibitors and have provided a number of antiinfective drugs. The purpose of this review is to highlight our efforts directed against HIV-1 integrase, with particular emphasis on the discovery and identification of potential drug candidates from natural product sources.

HIV-1 integrase assays

The assays used to screen natural products have been described in detail in previous reports (6-10). The



Step	Coupled	Assembly	Strand transfer
	Add compound	**Add compound**	
1	Add integrase to **Unprocessed** donor (30 min at 37 ºC)	Add integrase to **Preprocessed** donor (30 min at 37 ºC)	Add integrase to **Preprocessed** donor (30 min at 37 ºC)
2		Remove unbound integrase (wash)	Remove unbound integrase (wash)
3	Add target substrate (30 min at 37 ºC)	Add target substrate (30 min at 37 ºC)	**Add compound** Add target substrate (30 min at 37 ºC)
4	Assay strand transfer products	Assay strand transfer products	Assay strand transfer products

Fig. 2. Schematic representation of recapitulations of various integrase assays.

experimental approach chosen to assay for inhibitors of HIV-1 integrase relied on the various reactions catalyzed by the enzyme. As shown schematically in Figure 2, it was possible to evaluate several reactions catalyzed by integrase separately, *i.e.*, assembly and strand transfer, as well as the coupled reaction, in a high-throughput manner. This approach allowed us to identify which integrase-mediated enzymatic reaction(s) was being inhibited. This approach was sufficiently robust that it was possible to fully automate these assays (11) and transfer this technology to our ultra-high-throughput screening group. Specifically, both the coupled and strand transfer assays were used for screening natural product extracts at various times.

Further evaluation of natural product inhibitors of integrase made use of a 3'-end-processing assay, as described by LaFemina *et al.* (12). A cellular single-cycle viral infectivity assay was reported by Hazuda *et al.* (13) and was also used to determine if inhibitors possessed any cellular activity.

Microbial isolation

Fungal and actinomycete strains were isolated from environmental sources following methods described in the literature (14-19). Microbial strains were isolated from environmental samples collected in more than 50 countries covering a broad geographical diversity. Europe, Asia, Africa and Central America contributed similarly, altogether accounting for nearly 75% of all the strains tested. The rest of the isolates tested came from samples collected in North and South America, as well as Oceania.

Most of the isolates tested were recovered from soil- or plant-derived samples. Living leaves, wood and bark, together with leaf litter and other decaying plant materials, provided more than 75% of the fungal isolates, with the rest coming from herbivore dung (9%), soil (6%), fungal fruiting bodies (4%) and other minor contributions from marine and freshwater samples, as well as lichens. Actinomycetes were preferentially isolated from soil samples (60%), followed by decaying plant material (34%), the rest coming from other sources (dung, marine and freshwater samples). Following microbial isolation, dereplication of redundant strains was carried out using macro- and micromorphological criteria, and, in the case of actinomycetes, chemotaxonomy by FAME (fatty acid methyl ester) analyses (20, 21).

Microbial fermentation

The microbial isolates selected for screening were cultivated in several different media and conditions to maximize secondary metabolite production. Cultures were first transferred from fresh slants into flasks containing a seed medium, and these seed cultures were used for inoculation of the production media, as described (22, 23). Each fungal isolate was grown in 3 or 4 different media. One medium was usually grain- or vermiculite-based and incubated statically for 14-21 days; the rest were liquid media that were incubated with agitation for 7, 14 or 21 days. For actinomycetes, each isolate was grown in 2-4 liquid media at 1 or 2 incubation periods

(4, 7 or 10 days), for a total of 3-6 conditions. The fermentations were carried out in 250-ml flasks containing 50 ml of the production medium. All the production media and conditions used in this screening were previously described (22, 23). Production cultures were extracted with methyl ethyl ketone (MEK), which was concentrated and used for screening (24). On occasion, the cultures were extracted with methanol. A final concentration of 2-18 µl/ml of whole broth equivalent (WBE = amount equivalent to the volume of fermentation broth), depending on the type of microorganism, was used for testing. Generally, all test samples, including an internal control, were dissolved in DMSO for testing in 96-well microtiter plates.

Screening of natural products

Our efforts to discover natural product inhibitors of HIV-1 integrase spanned a period of 5 years (1993-1998) and was comprehensive. Almost 200,000 MEK extracts of microbial fermentations, either freshly prepared or stored as a DMSO solution in a library format, prepared from more than 50,000 different strains (Table I), were screened. About 9% of the fermentations prepared were also extracted with MeOH and tested. About 60% of the strains tested were fungi, 40% were actinomycetes and the rest were unicellular bacteria. A subset of representative data is shown in Table I. About 0.3% of the extracts tested were selected for further study following primary screening, corresponding to 1.2% of the strains tested. The hit rate was significantly higher for fungi than for actinomycetes or bacteria, at the level of both extracts and strains. It should be noted that the number of active extracts is rather close to the number of active strains (385 *vs.* 330), suggesting that most of the strains tested were active only in 1 fermentation condition.

Phylogenetic diversity of the producing organisms

A taxonomic analysis of the microorganisms producing inhibitors of HIV-1 integrase (Table II) reveals a remarkable phylogenetic diversity. Most of the producing strains were fungi, with representation of only 2 actinomycetes, those producing isocomplestatin (*Streptomyces* sp.) and integramycin (*Actinoplanes* sp.). Among the fungi, all the producing strains were ascomycetes anamorphs, except for 2 basidiomycetes (those producing hispidin/caffeic acid and xerocomic acid). Within the ascomycetes, there was a broad representation of different orders, with 6 strains being members of the Eurotiales (producers of xanthoviridicatins, altenusin, deoxyfunicone, ophiobolins, terphenyllin, rugulosin), 6 Hypocreales (integracides, integramides, equisetin, bis[naphtho-γ-pyrones], 8-*O*-methylanthrogallol and aquastatin A), 2 Diaporthales (integracin and cytosporic acid), 2 Pleosporales (integrastatins, phomasetin), while the rest were members of Xylariales (integric acid) or had an uncertain classification (roselipins, integrasone, exophillic acid).

Interestingly, this distribution does not reflect the taxonomic diversity of the organisms screened. As shown in Table I, actinomycetes were screened in similar number to fungi with quite a different output. It is unclear why fungal strains were more productive than actinomycetes or unicellular bacteria in producing integrase inhibitors, but this difference was also observed in other screening efforts seeking inhibitors of other enzymes in different therapeutic areas (25). Within the fungi, the prevalence of Eurotiales and Hypocreales in the list of producing strains does not reflect any bias towards testing higher numbers of these fungi in the screening. Actually, members of the Xylariales, Diaporthales and Pleosporales were screened in much larger numbers than Eurotiales and Hypocreales. However, the higher incidence of anamorphic ascomycetes *versus* basidiomycetes among the producing organisms indeed correlates with the fact that most of the strains tested were members of the former group.

The apparently higher capacity of Eurotiales and Hypocreales as producers of inhibitors of HIV-1 integrase correlates well with the known potential of these groups to produce bioactive secondary metabolites, as described in the literature (26). However, it should be noted that some of the most interesting compounds in the list (integric acid, complestatins) were not produced by members of these two groups. This is not unique to this screening effort. Similar observations have been made in other screening campaigns against other targets as well (25). One lesson learned from this is that microbial groups yielding a higher number of hits are not necessarily those producing the most interesting compound in a specific screen. Another lesson learned was that it was strategically more productive to maximize the biological diversity of the organisms screened to increase the chances of finding novel leads.

Table I: Summary of screening efforts (1994-1996) against HIV-1 integrase.

	Strain			Extract		
Microorganism	Tested	Hits	Hit rate (%)	Tested	Hits	Hit rate (%)
Fungi	15,711	253	1.6	51,596	282	0.55
Actinomycetes	12,324	76	0.6	75,844	102	0.13
Bacteria	387	1	0.25	1,473	1	0.07
Total	28,422	330	1.2	128,913	385	0.30

Table II: HIV-1 integrase inhibitors discovered by screening microbial extracts.

Inhibitor	Producing strain	Fungal order
8-*O*-Methylanthrogallol	*Cylindrocarpon ianthotele*	Hypocreales
Altenusin	*Talaromyces flavus*	Eurotiales
Aquastatin A	*Fusarium aquaeductum*	Hypocreales
Bis(naphtho-γ-pyrones)	*Fusarium* sp.	Hypocreales
Complestatins/chloropeptin	*Streptomyces* sp.	-
Cytosporic acid	*Cytospora* sp.	Diaporthales
Deoxyfunicone	*Penicillium* sp.	Eurotiales
Equisetin	*Fusarium heterosporum*	Hypocreales
Exophillic acid	*Exophiala pisciphila*	-
Hispidin/caffeic acid	*Inonotus tamaricis*	Hymenochaetales
Integracides	*Fusarium* sp.	Hypocreales
Integracins	*Cytonaema* sp.	Diaporthales
Integramides	*Dendrodochium* sp.	Hypocreales
Integramycin	*Actinoplanes* sp.	-
Integrasone	Undetermined fungus	-
Integrastatins	*Ascochyta* sp./undetermined fungus	Pleosporales
Integric acid	*Xylaria* sp.	Xylariales
Ophiobolins	*Neosartorya* sp.	Eurotiales
Phomasetin	*Phoma* sp.	Pleosporales
Roselipin 2A/2B	Undetermined fungus	-
Rugulosin	*Penicillium islandicum*	Eurotiales
Terphenyllin/hydroxyterphenyllin	*Aspergillus candidus*	Eurotiales
Xanthoviridicatins E/F	*Penicillium chrysogenum*	Eurotiales
Xerocomic acid	*Xeromphalina junipericola*	Agaricales

Natural product inhibitors

Prior to fractionation, the active extracts were tested in secondary assays to eliminate extracts that were non-specific intercalators of DNA or inhibitors of either DNase or reverse transcriptase enzymatic activities. Extracts that exhibited a suitable inhibitory ratio selective for HIV-1 integrase were selected for fractionation. In general, a 2-step chromatographic procedure was applied for the purification of HIV-1 inhibitors from MEK extracts of microbial cultures. Extracts were first chromatographed on Sephadex LH20, followed by reversed-phase HPLC, leading to pure natural products, which are detailed in this review. Later in the screening campaign, when the extracts were screened in a library format, the same chromatographic methods were applied in a parallel mode, which significantly improved the throughput and expedited the isolation and characterization of pure natural products. Natural product inhibitors have been classified into two major groups based on the microbial source, namely eukaryotic and prokaryotic metabolites, and these were further grouped into broad structural classes.

Eukaryotic metabolites

As discussed earlier, fungal sources were the most productive in affording natural products that were inhibitors of HIV-1 integrase. More than 95% of the identified inhibitors were derived from fungal sources.

1. Decalin-containing fungal metabolites

Inhibitors of this class possess a decalin unit substituted with various groups, such as hydrophilic tetramic acid, pyrrolidinone, vinyl aldehyde, carboxyl and various alkyl groups, and are represented by equisetin, phomasetin, integric acid, cytosporic acid, australifungin, coprophilin, nalanthalide and oteromycin. Biogenetically, all of these compounds, except for nalanthalide, are derived from the polyketide pathway.

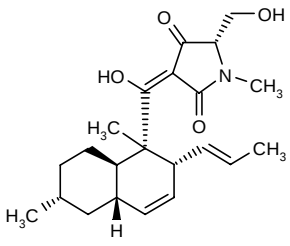
Equisetin

Equisetin (**1**), a tetramic acid-containing *trans*-decalin, was isolated from *Fusarium heterosporum* (27, 28). This was the first inhibitor discovered in the screening program in 1993, and was possibly the first natural product HIV-1 integrase inhibitor discovered anywhere. It inhibited the 3'-end processing activity of recombinant HIV-1 integrase, the coupled reaction and strand transfer with IC_{50} values of 12.5, 7.5 and 15 μM, respectively (Table III). Additionally and not unexpectedly, equisetin inhibited integrase activity when a full-site preintegration complex (PIC) isolated from HIV-1-infected cells was used, with an IC_{50} value of 25 μM. Furthermore, equisetin inhibited the disintegration activity of HIV-1 integrase, giving an IC_{50} of 20-25 μM. An analogue of equisetin, 5'-epiequisetin (**2**), synthesized using base catalysis, was equally active against the coupled reaction and strand transfer (IC_{50} = 10 and 15 μM, respectively) (27).

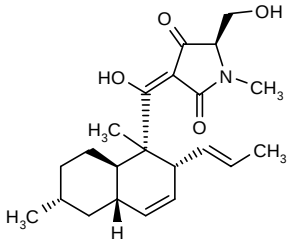
Table III: HIV-1 integrase-inhibitory activity of natural products and analogues.

Compound	Coupled reaction (IC_{50}, μM)	Strand transfer (IC_{50}, μM)	Compound	Coupled reaction (IC_{50}, μM)	Strand transfer (IC_{50}, μM)
1 (Equisetin)	7.5	15	**60**	3	12
2 (5'-Epiequisetin)	10	20	**61**	> 50	> 50
3 (Phomasetin)	10	18	**62**	> 50	> 50
4 (5'-Epiphomasetin)	12	20	**63**	8	> 50
5	15	20	**64**	> 50	> 50
6 (Integric acid)	3	10	**65**	> 50	> 50
7/8	11	50	**66**	> 50	> 50
9	100	> 100	**67**	> 50	> 50
10	45	> 100	**68**	1.7	11.5
11	73	> 100	**69**	23	> 50
12	> 100	> 100	**70**	18	> 50
13	3	> 100	**71** (Integramide A)	17	60
14	5	> 100	**72** (Integramide B)	10	60
15	5	> 100	**73+74** (Roselipin 2A/2B)	8.5	NT
16	12	> 100	**75** (Integrasone)	NT	41
17	30	> 100	**76** (Integracin A)	3.2	32
18	75	> 100	**77** (Integracin B)	6.1	17
19	24	> 100	**78** (Integracin C)	3.5	88
20	10	> 100	**79** (Exophillic acid)	NT	68
21 (Cytosporic acid)	NT	20	**80** (Aquastatin A)	NT	50
22 (Australifungin)	NT	20	**81** (Xanthoviridicatin A)	6	> 100
23 (Australifunginol)	NT	> 200	**82** (Xanthoviridicatin B)	5	> 100
24 (Coprophilin)	10	25	**83** (Isochaetochromin B_1)	2	12
25 (Nalanthalide)	10	25	**84** (Isochaetochromin B_2)	2	12
26 (Oteromycin)	25	50	**85** (Isochaetochromin D_1)	1	4
27 (Epiophiobolin K)	29	> 120	**86** (Oxychaetochromin B)	3	9
28 (Epiophiobolin C)	30	> 120	**87**	50	> 100
29 (Ophiobolin A)	48	> 120	**88**	60	> 100
30 (Ophiobolin B)	21.5	> 120	**89**	40	> 100
31 (Ophiobolin C)	6.7	33	**90**	80	> 100
32 (Ophiobolin H)	30	> 120	**91**	40	> 100
33 (Ophiobolin K)	23	> 120	**92**	8	> 100
34 (Integracide A)	4	9	**93** (Integrastatin A)	0.6	1.1
35 (Integracide B)	82	> 100	**94** (Integrastatin B)	1.04	2.5
36 (Integracide C)	> 100	> 100	**95** (8-O-Methylanthrogallol)	6	22
37 (Integracide D)	50	> 100	**96** (Rugulosin)	19	25
38	5	15	**97** (Deoxyfunicone)	11	> 140
39	5	14	**98** (Hispidin)	2	24
40	5.6	5.6	**99** (Caffeic acid)	2.8	24
41	3.1	4.8	**100** (CAPE)	NT	18.9
42 (Clavaric acid)	47	85	**101**	> 100	> 100
43	70	> 100	**102** (Xerocomic acid)	1.1	4.4
44	68	> 100	**103** (Altenusin)	19	25
45	50	> 100	**104** (Terphenyllin)	17.7	47.7
46	50	> 100	**105** (Hydroxyterphenyllin)	2.8	12.1
47	75	> 100	**106** (Integramycin)	3	4
48	25	40	**107**	6.1	6.1
49	> 50	> 50	**108**	6	30-40
50	48	> 50	**109** (Isocomplestatin)	0.2	4
51	> 50	> 50	**111** (Chloropeptin I)	0.4	5
52	> 50	> 50	**112** (Complestatin A)	0.8	12.5
53	> 69	> 69	**113** (Complestatin B)	1.7	12.5
54	> 80	> 80	**114**	0.6	8
55	> 80	> 80	**115**	0.3	6
56	> 80	> 80	**116**	3.8	45
57	> 80	> 80	**117**	7.8	86
58	> 80	> 80	**118**	28	> 100
59	3	12			

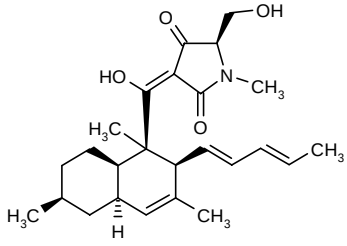
NT: not tested.



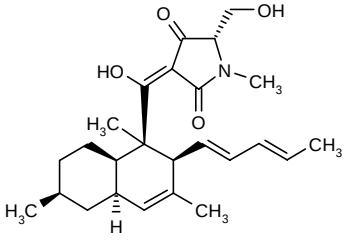
(**1**) Equisetin



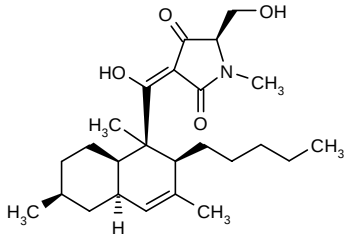
(**2**) 5'-Epiequisetin



(**3**) Phomasetin



(**4**) 5'-Epiphomasetin



(**5**)

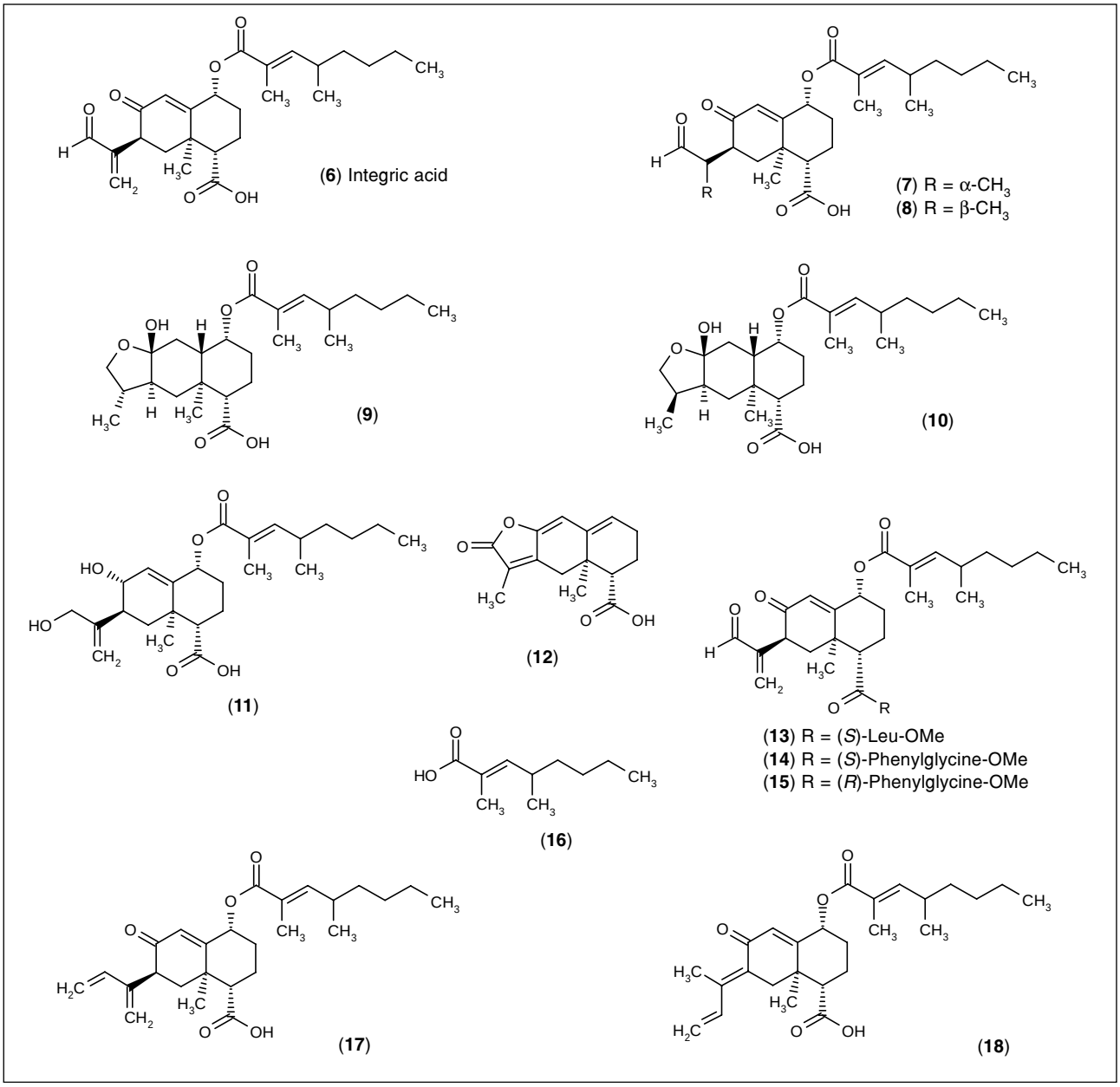
Phomasetin

Phomasetin (**3**), an enantiomeric homologue of equisetin, was isolated from a *Phoma* sp. (27, 28) concurrently with equisetin. Aside from the opposite stereochemistry, it differs from equisetin by having an additional methyl group at C-4 of the decalin unit and an extra olefin in the alkyl chain. Phomasetin was as active as equisetin in the coupled reaction and strand transfer assays, with IC_{50} values of 10 and 18 μM, respectively, despite their enantiomeric relationship, indicating a nonstereospecific inhibition, potentially due to the binding of tetramic acid at either the magnesium- and/or zinc-binding domains of integrase. While this suggestion is plausible, it may not be completely accurate, since simple tetramic acids were inactive. C-5'-epimerization (*e.g.*, 5'-epiphomasetin, **4**) and hydrogenation (*e.g.*, tetrahydrophomasetin, **5**) did not have any effect on the activity of these derivatives against the coupled reaction and strand transfer.

Integric acid

Integric acid (**6**), a decalin that is substituted with a carboxyl, a vinyl aldehyde and an octanoate, was isolated from a *Xylaria* sp. (29). It was moderately more potent than either equesetin or phomasetin, with IC_{50} values of 10, 3 and 10 μM, respectively, in the 3'-end processing, the coupled reaction and the strand transfer assays (Table III). Similar to equisetin, integric acid also inhibited full-site integration (IC_{50} = 30 μM) and wild-type disintegration (IC_{50} = 5 μM).

Integric acid was studied further in an effort to understand the structure-activity relationships (SAR). Derivatives of integric acid were only tested in the coupled and strand transfer assays to determine their inhibitory potential. While most of these compounds exhibited activity in the coupled assay (Table III) and the activity of many compounds (*e.g.*, **7, 8, 13-16** and **20)** was comparable to that of integric acid, none retained strand transfer activity at 100 μM, except for the dihydro compounds **7** and **8**. The latter compounds exhibited weak inhibition, with IC_{50} values of 50 μM. Vinyl group reduction (*e.g.*, **7** and **8**) led to a minor reduction in potency in the coupled assay, although the reduction in both the aldehyde and the ketone groups (**11**), and removal of the aldehyde group either by cyclization (**9** and **10**) or by homologation (**17** and **18**), caused a significant decrease in activity in the coupled assay. The integrase-inhibitory activity was considerably diminished upon elimination of the side-chain (**12** *versus* **6**), but surprisingly, the side-chain acid **16** retained most of the inhibitory activity in the coupled assay, indicating that a fatty acid binding to either enzyme or DNA could contribute to the activity. (N.B. Surprisingly, we did not isolate any fatty acids from any of the extracts that were selected for isolation, suggesting that they may have been screened out by the secondary assays.) The activity against the coupled reaction was not affected by blocking the carboxy group with amides (**13-15**), while the less constrained dimer **20** was 2.4-fold more active in the coupled assay than the constrained dimer **19** (Table III). These data suggest that certain modifications are tolerated for the coupled reaction, but the acid groups at C-4, the vinyl aldehyde at C-11 and the ester groups at the C-1 are required for strand transfer activity. The observation that the coupled assay, requiring both catalytic activities, is affected to a lesser degree by these modifications suggests different SAR requirements for the cleavage, assembly and stand transfer reactions of the enzyme.

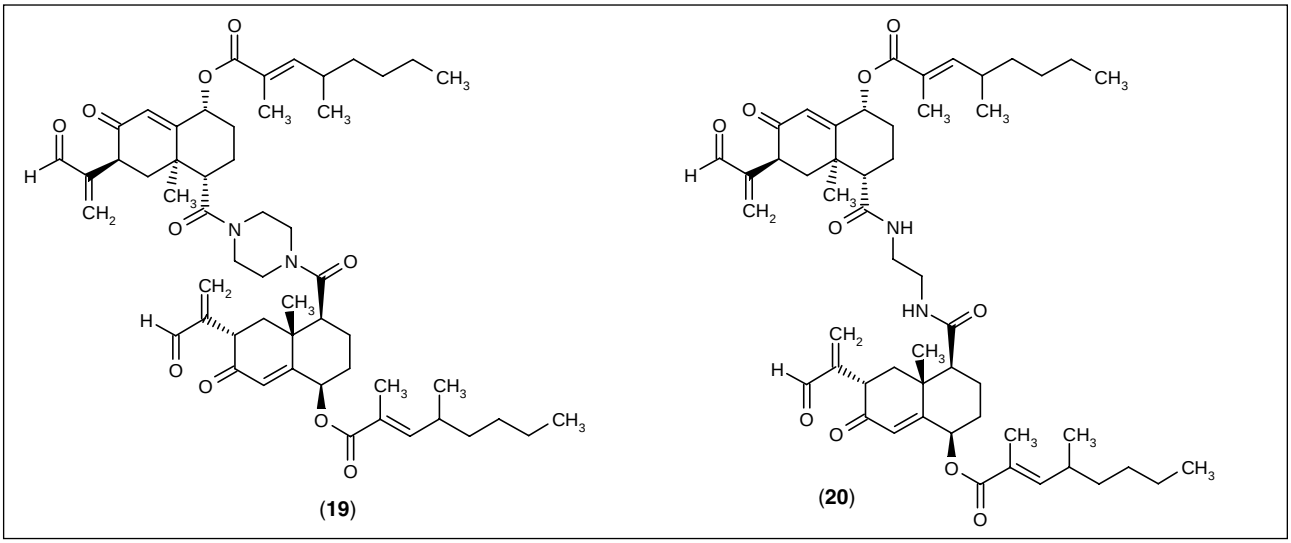


Cytosporic acid/australifungin/australifunginol

Cytosporic acid (**21**), a bisalkyldecalinenonecarboxylic acid, was isolated from a *Cytospora* sp., an endophyte which was isolated from leaf litter of *Manilkara bidentata*. It inhibited the strand transfer reaction with an IC_{50} value of 20 μM. Australifungin (**22**) and australifunginol (**23**), two structurally related compounds that were originally discovered as antifungal agents, were also tested in the strand transfer assay. While australifungin (IC_{50} = 20 μM) exhibited identical activity to cytosporic acid, australifunginol was completely inactive at 200 μM. Structural comparison suggested that the chelating β-ketoaldehyde functionality of australifungin and the negatively charged carboxyl group of cytosporic acid may be required for strand transfer activity.

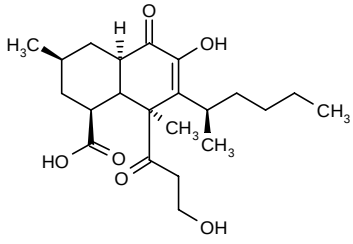
Coprophilin

Coprophilin (**24**) was discovered from an unidentified dung-inhabiting fungus as an anticoccidial agent (*Eimeria tenella* MIC = 1.5 μM) in 1998 (30) and was evaluated against HIV-1 integrase due its structural similarities to the decalin-containing integrase inhibitors, *e.g.*, integric acid and equisetin. It has been reported (31) to inhibit the coupled reaction and strand transfer with IC_{50} values of 10 and 25 μM, respectively, which are essentially similar to other decalin-containing compounds.

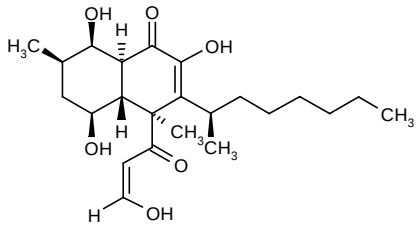


(**19**)

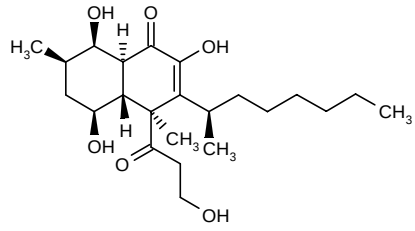
(**20**)



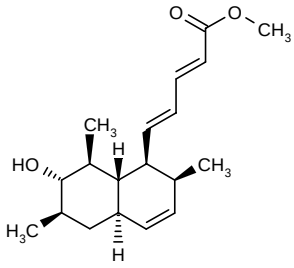
(**21**) Cytosporic acid



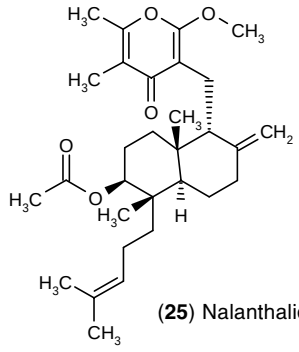
(**22**) Australifungin



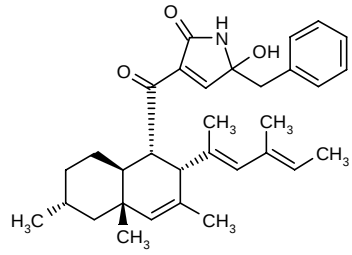
(**23**) Australifunginol



(**24**) Coprophilin



(**25**) Nalanthalide



(**26**) Oteromycin

Nalanthalide

Nalanthalide (**25**) is a decalin-containing diterpenoid pyrone that was originally reported in 2001 (32) as a blocker of the voltage-gated potassium (Kv1.3) channel (IC_{50} in various assays ranging from 0.5 to 3.9 μM). It is produced by *Nalanthamala* sp. While it was not directly discovered in the HIV-1 integrase screening assay, like other decalin-containing compounds it displayed IC_{50} values of 10 and 25 μM, respectively, in the coupled and strand transfer assays (31).

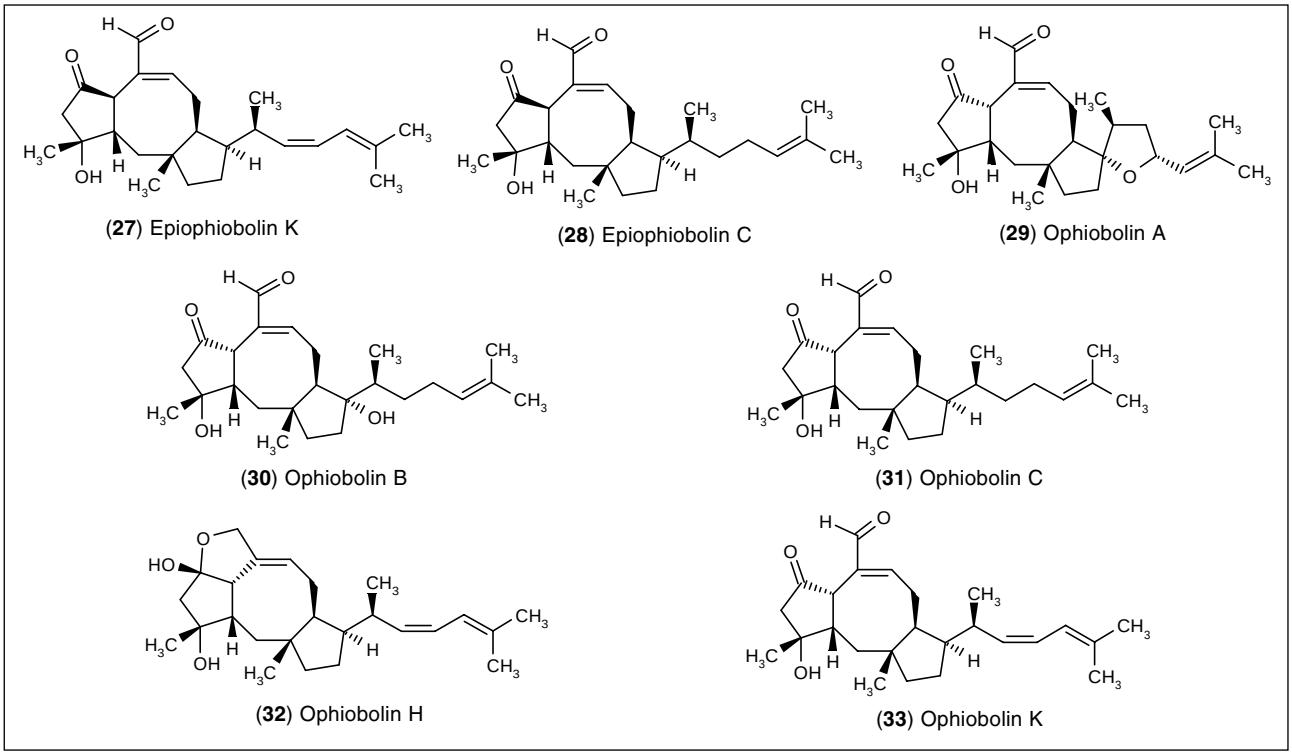
Oteromycin

Oteromycin (**26**), an alkyl-substituted decalin with a pyrrolidinone ring, was isolated from two unidentified fungi in 1995 (33) as an endothelin receptor antagonist (ET_B IC_{50} = 2.5 μM) and was evaluated in the coupled and strand transfer assays because of its structural similarities with the decalin-based inhibitors isolated in the integrase program. This compound inhibited the coupled and strand transfer reactions with IC_{50} values of 25 and 50 μM, respectively, and was therefore only approximately 3-fold less active than equisetin (28).

2. Sesterterpenoids

Ophiobolins

Epiophiobolin K (**27**) and epiophiobolin C (**28**) were produced by a strain of *Neosartorya* sp. (31). A number



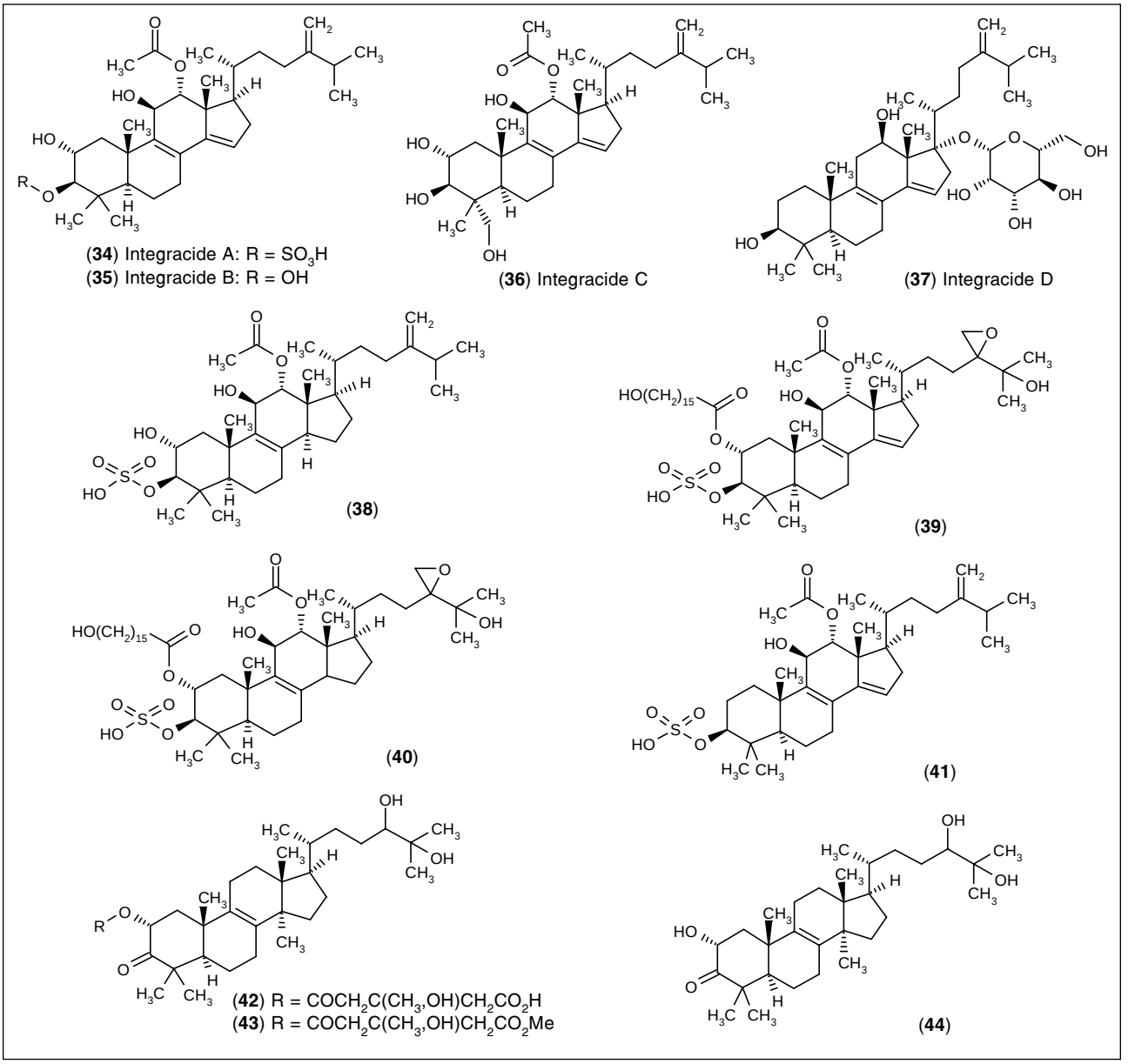
of readily available ophiobolins (*e.g.*, ophiobolins A [**29**], B [**30**], C [**31**], H [**32**] and K [**33**]) (34, 35) were evaluated in the integrase assays. Epiophiobolins K and C have been shown to inhibit HIV-1 integrase with IC_{50} values of 29 and 33 μM, respectively, in the coupled assay, but both were found to be inactive in the strand transfer assay (IC_{50} > 120 μM). Ophiobolins A, B, C, H and K were similarly shown to be effective in the coupled assay, exhibiting IC_{50} values of 48, 21.5, 6.7, 30 and 23 μM, respectively. Of these, only ophiobolin C was active in the strand transfer assay, with an IC_{50} value of 33 μM. The other ophiobolins were less active (IC_{50} > 120 μM), indicating the importance of the *cis*-fused A/B ring and potentially the dehydro side-chain of ophiobolin C for strand transfer activity. Ophiobolins have been widely reported to possess activity against other biological targets and this was recently reviewed (36).

3. Triterpenoids

Integracides

Integracides (**34-37**), members of the 4,4-dimethylergostane tetracyclic triterpenoid family, were isolated from the filamentous fungus *Fusarium* sp. (37) as inhibitors of HIV-1 integrase. Related natural products (**38-44**) were obtained from the Merck natural products collection discovered in various other programs as inhibitors of elastase (*e.g.*, **38-40**), fungi (*e.g.*, **41**) and Ras protein farnesyltransferase (*e.g.*, **42-44**). Compounds **45-70** were prepared by chemical modification of integracide B (**35**) (38).

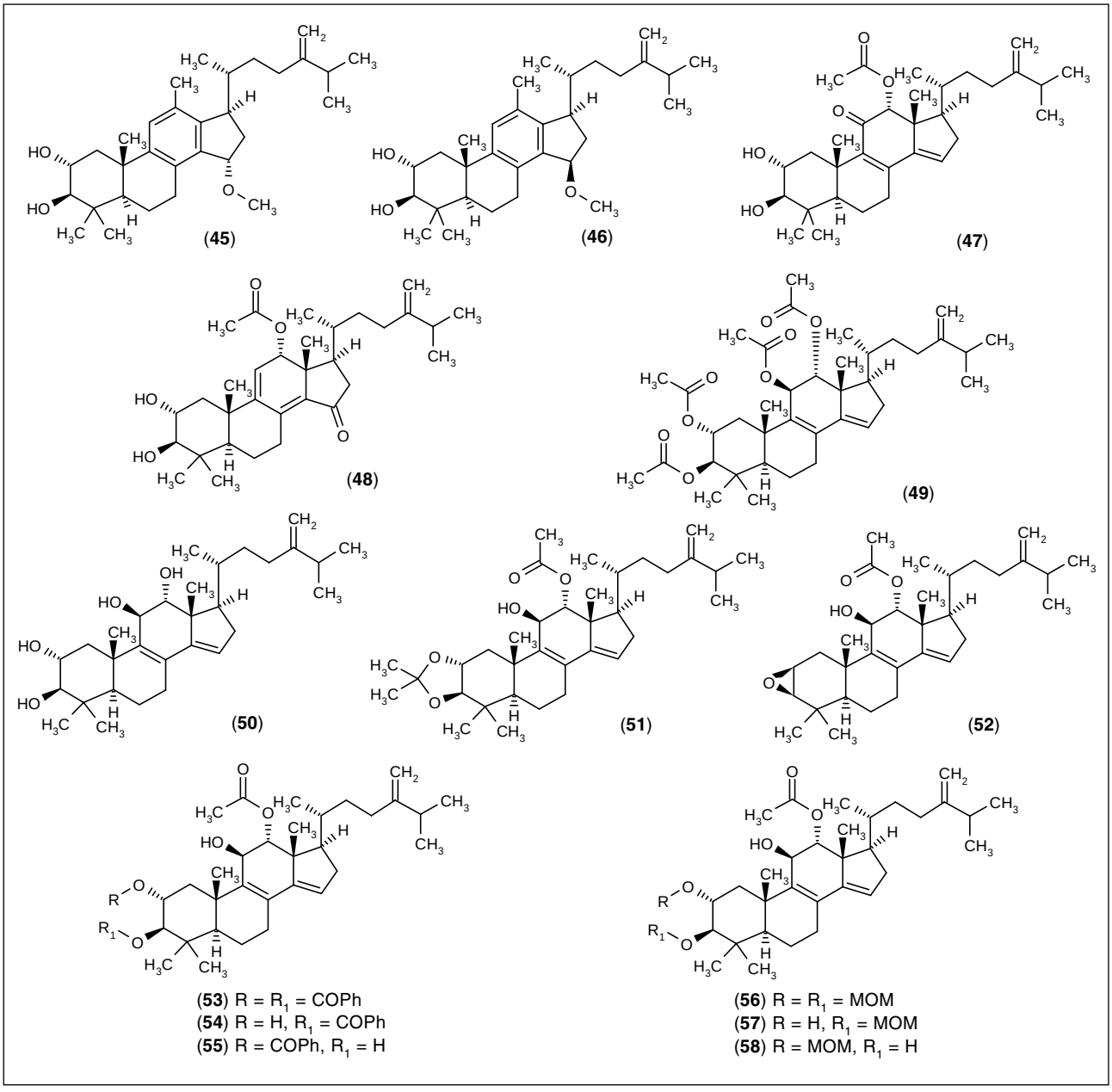
Integracide A (**34**), the lead compound in this series of inhibitors, inhibited the coupled and strand transfer reactions with IC_{50} values of 4 and 9 μM, respectively (37). Like integracide A, all natural products with a sulfate ester at the C-3 position (*e.g.*, **38-41**) were essentially equipotent and exhibited IC_{50} values of 5-6 μM in the coupled assay (Table III). The 2-deoxy compound (**41**) showed slightly better activity in this assay (IC_{50} = 3.2 μM), while a bulky ester group (*e.g.*, **39** and **40**) at C-2 had no impact on inhibitory activity in the coupled assay (37, 38). While the ability of these compounds to inhibit the coupled reaction was essentially identical, their ability to inhibit the strand transfer reaction was significantly different. The 2-deoxy compound (**41**) was the best inhibitor (IC_{50} = 4.8 μM) of the strand transfer reaction in this series, with 2-fold better potency than integracide A. Compound **39** was slightly less active (IC_{50} = 14 μM) than **34** in the strand transfer assay, whereas the dihydro analogue **40** was approximately 3-fold more potent than **39**. The latter compound was equipotent in both the coupled and strand transfer assays. The dihydro analogue **38** (IC_{50} = 15 μM) was slightly less active than **34** (IC_{50} = 9 μM) in the strand transfer assay. The SAR of these compounds indicated that oxygenation (*e.g.*, C-25 hydroxy and 23,24-epoxide in **39** and **40**) of the C-17 side-chain plays no role in the activity of these compounds. Clavaric acid (**42**), a protein farnesyltransferase inhibitor, was significantly less active



than the sulfated compounds and showed IC_{50} values of 47 and 85 μM in the coupled and the strand transfer assays, respectively (37, 38).

The desulfated compounds **35**, **36** and **37** were significantly less active in the coupled assay, with IC_{50} values of 82, > 100 and 50 μM, respectively (Table III). These compounds were completely inactive at 100 μM in strand transfer assays, indicating that the negatively charged sulfate group is critical for strand transfer activity. Other neutral compounds, such as methyl ester **43**, clavarinone **44** and compounds **45-58**, were either significantly less active or inactive in both assays, with the exception of the 15-keto analogue **48**, which was shown to inhibit the coupled reaction and strand transfer with IC_{50} values of 25 and 40 μM, respectively (38).

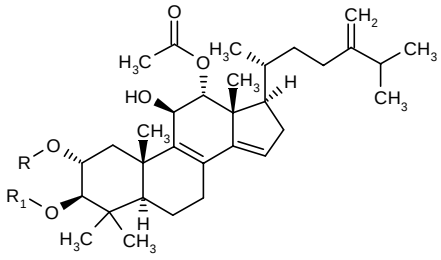
The activities of the hemisuccinate derivatives **59** and **60** containing a free carboxyl or the glycine ester derivatives **68-70** containing free amino groups, particularly the bisamino derivative **68,** appeared similar to the sulfated esters. These compounds exhibited variable levels of activity in the coupled and strand transfer assays. Both the 2-hemisuccinate **59** and 3-hemisuccinate **60** exhibited indistinguishable activities. Each displayed identical IC_{50} values of 3 and 12 μM in the coupled and strand transfer assays, respectively. The monoamino esters **69** and **70** were both weakly active in the coupled assay, but were essentially inactive in the strand transfer assay at 50 μM. However, the bisamino derivative **68**, with two net positive charges, was reported to be one of the most potent compounds in this series in the coupled assay



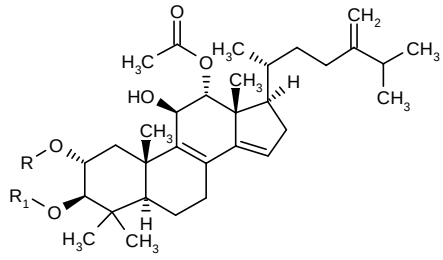
(IC_{50} = 1.7 μM), and it also displayed good activity in the strand transfer assay (IC_{50} = 11.5 μM). As observed earlier, the neutral compounds, *i.e.*, compounds with a protected acid group (**61** and **62**) or protected amino groups (**64-66**), were completely inactive in both assays. The hydroxymate derivative **63** showed only weak activity in the coupled assay, displaying an IC_{50} of 8 μM, and it was inactive in the strand transfer assay. The activity of the charged compounds was rationalized by their potential to interact with bivalent metal ions (*e.g.*, Mg^{2+}) at the active site, but the lack of activity of the hydroxymate **63** would contradict this hypothesis.

These derivatives were selective for HIV-1 integrase *versus* standard DNAase assays (IC_{50} > 50 μM), except for the bisamino compound **68,** which inhibited DNAse with an IC_{50} value of 47 μM.

Integracide A and compounds **38** and **59** each inhibited the 3'-end processing reaction of HIV-1 integrase with IC_{50} values of 5 μM. The dihydro 2-ester and 3-sulfate ester-containing compound **40** displayed an IC_{50} value of 2 μM in the 3'-end processing assay, whereas the dehydro analogue **39** was 5-fold less active (IC_{50} = 10 μM) (37, 38). Compounds **38** and **40** inhibited assembly formation with IC_{50} values of 10 and 12 μM, respectively. A few of these compounds, such as **34**, **38** and **40,** were tested for



(**59**) R = H, R_1 = $COCH_2CH_2CO_2H$
(**60**) R = $COCH_2CH_2CO_2H$, R_1 = H
(**61**) R = H, R_1 = $COCH_2CH_2CO_2CH_3$
(**62**) R = $COCH_2CH_2CO_2CH_3$, R_1 = H
(**63**) R = $COCH_2CH_2CONHOH$, R_1 = H



(**64**) R = R_1 = $COCH_2NH$-Boc
(**65**) R = $COCH_2NH$-Boc, R_1 = H
(**66**) R = R_1 = $COCH_2NH$-Fmoc
(**67**) R = $COCH_2NH$-Fmoc, R_1 = H
(**68**) R = R_1 = $COCH_2NH_2$.TFA
(**69**) R = $COCH_2NH_2$.TFA, R_1 = H
(**70**) R = H, R_1 = $COCH_2NH_2$.TFA

their effect against the PIC. These compounds exhibited IC_{50} values of 50, 30 and 50 μM, respectively, in the PIC assay.

The cell-based activity of a number of key compounds in the series (*e.g.*, **34**, **38-40**, **59**, **60**) has been reported. In a multiple-cycle antiviral assay using HIV-1-infected H9 T-lymphoid cells, **34**, **38**, **39**, **40**, **59** and **60** were reported to exhibit CIC_{95} values of 25, 50, 6, > 50, 6 and 6 μM, respectively. However, they were cytotoxic at about the same concentrations and thus did not exhibit any therapeutic window (37, 38).

4. Acyclic peptides

Integramides

Integramides A (**71**) and B (**72**) are nonribosomal linear peptides consisting of 9 C_α-methylamino acids and were isolated from the fungus *Dendrodochium* sp. (39). Integramide A displayed IC_{50} values of 17 and 60 μM, respectively, in the coupled and strand transfer assays and was slightly less active than the homologue integramide B, which exhibited respective IC_{50} values of 10 and 60 μM in these assays. These compounds were toxic and therefore their cell-based antiviral activity could not be ascertained (39).

5. Long-chain polyketide glycosides

Roselipins

Roselipins 2A (**73**) and 2B (**74**) are propionate-derived polyketides possessing alternating methyl substitutions; esterified with D-arabinitol/D-mannose at one of the termini and containing an acetylglucoside, they were isolated from an unidentified fungus. These compounds were active in the coupled assay, with an IC_{50} value of 8.5 μM (31), but were not of further interest.

6. Bicyclic epoxypolyketides

Integrasone

Integrasone (**75**), an epoxydihydroxycyclohexenyl γ-lactone, was isolated from an unidentified fungus and inhibited the strand transfer reaction with an IC_{50} of 41 μM. Like integric acid, the aliphatic chain of integrasone may play a significant role in the integrase-inhibitory activity (40).

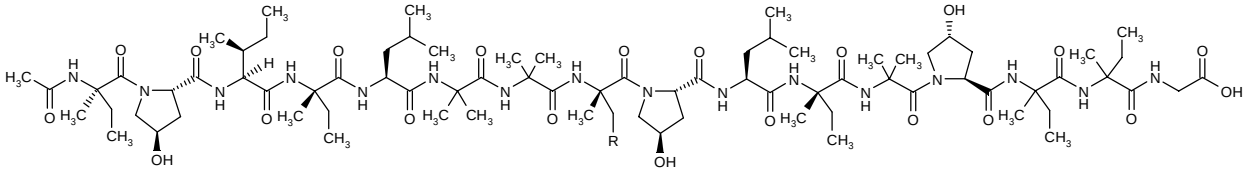
7. Alkyldihydroxybenzoic acids

Integracins

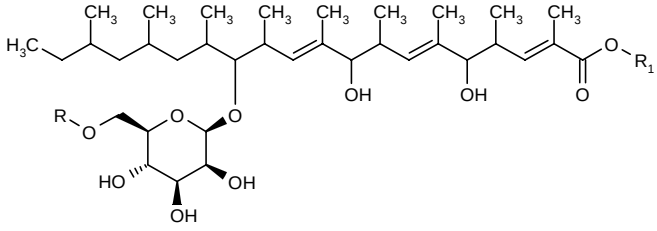
Integracins A (**76**), B (**77**) and C (**78**), isolated from *Cytonema* sp., are 6-alkyl-2,4-dihydoxybenzoic acids that are esterified with an alkyl-3,5-dihydroxybenzene. These compounds inhibited the coupled reaction with IC_{50} values of 3.2, 6.1 and 3.5 μM, respectively. Like other phenolics, integracins A-C were 10-30-fold less active in the strand transfer assay than the coupled assay, exhibiting IC_{50} values of 32, 17 and 88 μM, respectively (41).

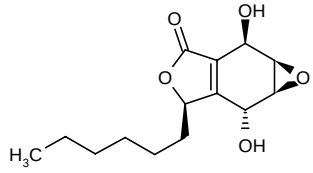
Exophillic acid

Exophillic acid (**79**), a symmetrically dimeric (6-nonyl-2,4-dihydroxybenzoic acid)glucoside, was produced by the fungus *Exophiala pisciphila* and inhibited strand transfer activity with an IC_{50} of 68 μM (42).

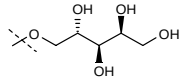
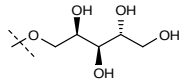


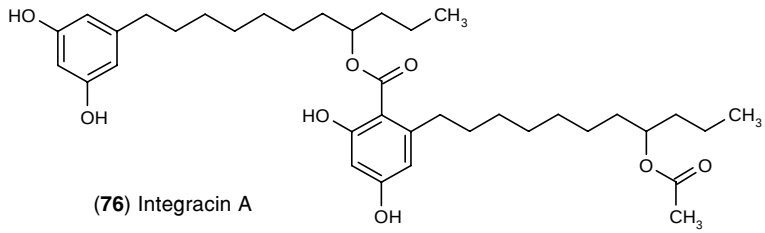
(**71**) Integramide A: R = H
(**72**) Integramide B: R = CH_3



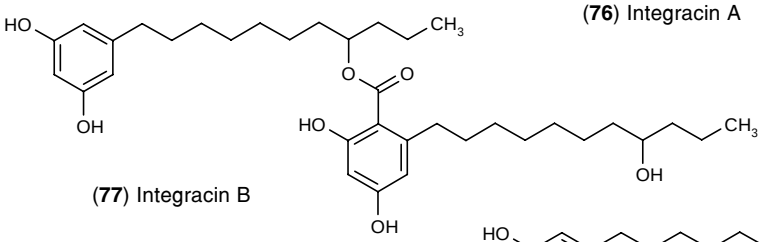


(**75**) Integrasone

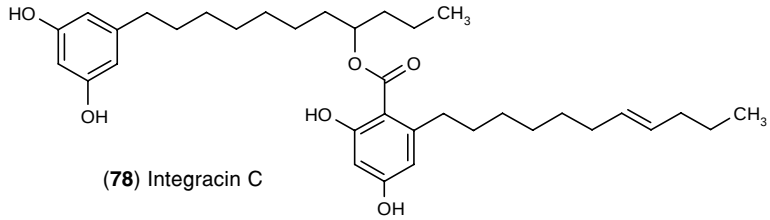
	R	R_1
(**73**) Roselipin 2A	Ac	
(**74**) Roselipin 2B	Ac	



(**76**) Integracin A



(**77**) Integracin B



(**78**) Integracin C

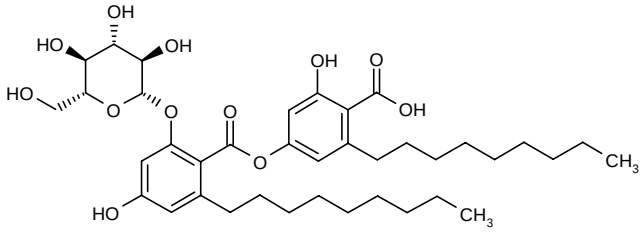
Aquastatin A

Aquastatin A (**80**), an asymmetric dimeric alkyl-2,4-dihydroxybenzoic acid galactopyranoside, was originally isolated from *Fusarium aquaeductuum* (42). Like other alkylbenzoic acids, it was also shown to inhibit strand transfer activity with an IC_{50} value of 50 μM (42).

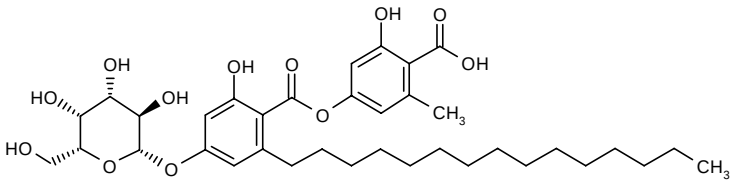
8. Polycyclic condensed phenolics

Xanthoviridicatins

Xanthoviridicatins E (**81**) and F (**82**) are isomeric aromatic polyketides that were isolated from *Penicillium chrysogenum* and were found to inhibit the coupled reac-



(**79**) Exophillic acid



(**80**) Aqustatin A

tion with IC_{50} values of 6 and 5 μM, respectively. These compounds were inactive in the strand transfer assay (IC_{50} > 100 μM) (43).

Bis(naphtho-γ-pyrones)

Isochaetochromins (**83-85**) and oxychaetochromin B (**86**), members of the chaetochromin family, were isolated from a *Fusarium* sp. (44). The atropisomeric isochaetochromins B_1 (**83**) and B_2 (**84**) were equally active against the coupled reaction and strand transfer, with IC_{50} values of 2 and 12 μM, respectively. Isochaetochromin D_1 (**85**), the dehydro analogue, was slightly more active and exhibited IC_{50} values of 1 and 4 μM, respectively, in the coupled and strand transfer assays. In contrast, oxychaetochromin B was slightly less active (IC_{50} = 3 μM) in the coupled assay, but was slightly more active (IC_{50} = 9 μM) than **83** and **84** in the strand transfer assay. Compounds **83** and **84** provided protection in a 24-h HIV-1 viral infectivity assay, with IC_{50} values of 3 and 2 μM, respectively. These compounds exhibited cytotoxicity at 50 μM in a 7-day infectivity assay and also in an MTT assay. The semisynthetic derivatives **87-92** of isochaetochromin B_2 were consistently less active in the coupled assay and were all inactive in the strand transfer assay (Table III), implicating the importance of the chelating phenolic groups present in the peri-position of the keto group, which plausibly interact with the bivalent metals at the active site of the enzyme (44).

Integrastatins

The two racemic natural integrastatins A (**93**) and B (**94**) were isolated from an unidentified sterile fungus and the latter compound was also produced by the endophytic fungus *Ascochyta* sp. (45). Integrastatin A displayed IC_{50} values of 0.6 and 1.1 μM in the coupled and strand transfer assays, respectively, while the aldehyde-containing integrastatin B was 2-fold less active in both assays (coupled reaction IC_{50} = 1.04 μM; strand transfer IC_{50} = 2.5 μM).

8-*O*-Methylanthrogallol

The naphthoquinone 8-*O*-methylanthrogallol (**95**) was isolated from a strain of *Cylindrocarpon ianthothele* and was reported to inhibit the coupled reaction and strand transfer with IC_{50} values of 6 and 22 μM, respectively (31).

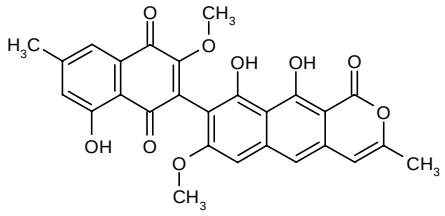
Rugulosin

Rugulosin (**96**), a highly complex dimeric naphthoquinone produced by a strain of *Penicillium islandicum,* displayed inhibitory activity in the coupled and strand transfer assays, with IC_{50} values of 19 and 25 μM, respectively (31).

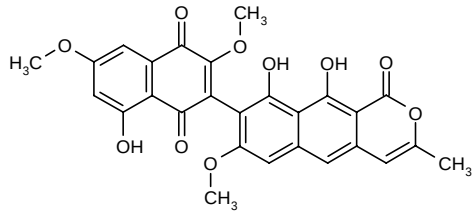
9. Benzo-γ-pyrones

Deoxyfunicone

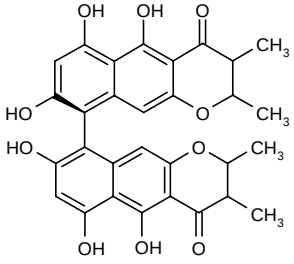
The benzo-γ-pyrone deoxyfunicone (**97**) is produced by a strain of *Penicillium* sp. and proved effective in the coupled assay (IC_{50} = 11 μM), while it was inactive in the strand transfer assay (IC_{50} > 140 μM) (31).



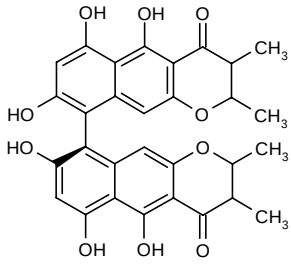
(**81**) Xanthoviridicatin E



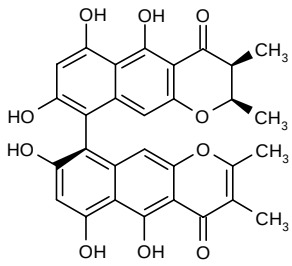
(**82**) Xanthoviridicatin F



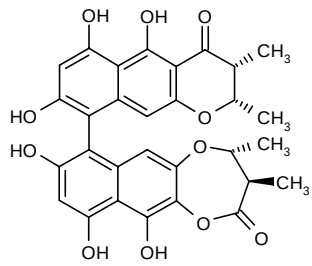
(**83**) Isochaetochromin B_1



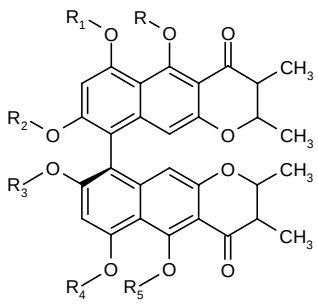
(**84**) Isochaetochromin B_2



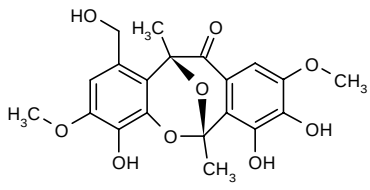
(**85**) Isochaetochromin D_1



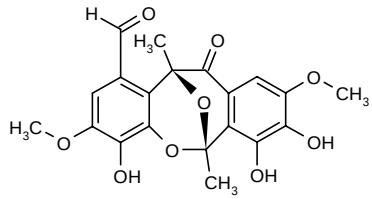
(**86**) Oxychaetochromin B



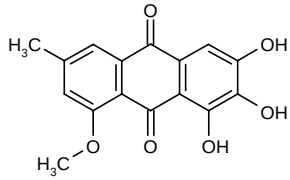
(**87**) R = R_1 = R_2 = R_3 = R_4 = R_5 = $COCH_3$
(**88**) R = R_1 = R_2 = R_3 = R_4 = R_5 = CH_3
(**89**) R = R_1 = R_2 = R_3 = R_5 = CH_3, R_4 = H
(**90**) R = R_1 = H, R_2 = R_3 = R_4 = R_5 = CH_3
(**91**) R = R_1 = R_2 = R_3 = CH_3, R_4 = R_5 = H
(**92**) R = R_2 = R_3 = R_5 = CH_3, R_2 = R_4 = H



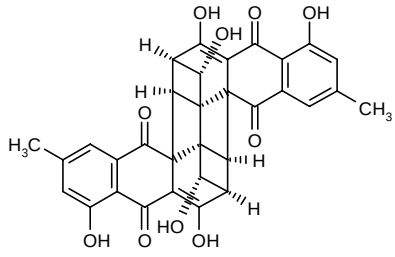
(**93**) Integrastatin A



(**94**) Integrastatin B



(**95**) 8-*O*-Methylanthrogallol



(**96**) Rugulosin

10. Compounds containing catechol units

Hispidin and caffeic acid

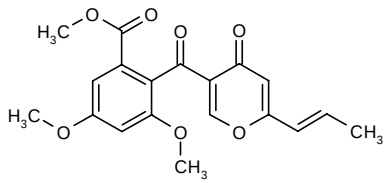
Hispidin (**98**) and caffeic acid (**99**), compounds containing a catechol unit, were produced by an unidentified fungus (31) and inhibited the coupled reaction with IC_{50} values of 2 and 2.8 μM, respectively. However, like many other catechols, these compounds were significantly less active in the strand transfer assay, both exhibiting IC_{50} values of 24 μM. In contrast, caffeic acid phenethyl ester (CAPE, **100**) was reported to inhibit strand transfer activity with an IC_{50} value of 18.9 μM, although it was a much weaker inhibitor of the cleavage reaction (IC_{50} = 220 μM) (46, 47). Blocking the phenolic groups with methyl groups (*e.g.*, **101**) completely abolished the activity in both the coupled and strand transfer assays, indicating the critical role of the catechol group.

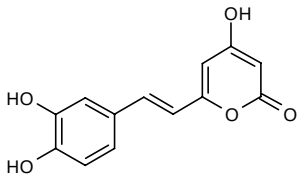
Xerocomic acid

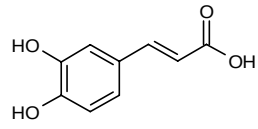
Xerocomic acid (**102**) was discovered from a strain of *Xeromphalina junipericola* and found to inhibit the coupled and strand transfer reactions with IC_{50} values of 1.1 and 4.4 μM, respectively (31).

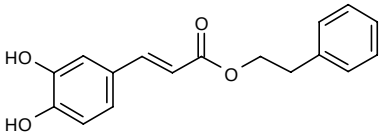
Altenusin

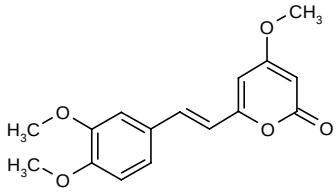
Altenusin (**103**), isolated from a strain of *Talaromyces flavus,* exhibited IC_{50} values of 19 and 25 μM, respectively, in the coupled and strand transfer assays (31).

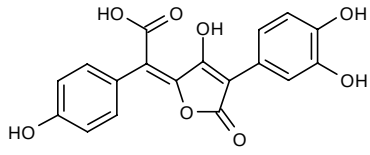

(**97**) Deoxyfunicone

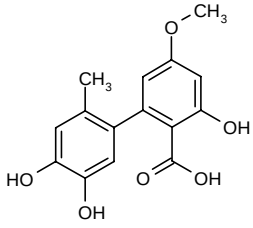

(**98**) Hispidin

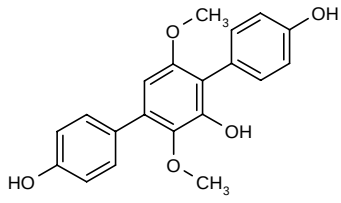

(**99**) Caffeic acid

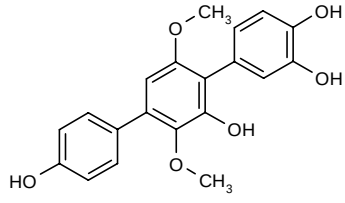

(**100**) CAPE


(**101**)


(**102**) Xerocomic acid


(**103**) Altenusin


(**104**) Terphenyllin


(**105**) Hydroxyterphenyllin

Terphenyllin and hydroxyterphenyllin

The linear triphenyls terphenyllin (**104**) and hydroxyterphenyllin (**105**) were isolated from a strain of *Aspergillus candidus* (31). The catechol-containing hydroxyterphenyllin was the more active of the two and exhibited IC_{50} values of 2.8 and 12.1 μM, respectively, in the coupled and strand transfer assays. As expected, terphenyllin, the desoxy compound, was less active in both assays, with respective IC_{50} values of 17.7 and 47.7 μM.

Prokaryotic metabolites

While extracts from prokaryotes constituted approximately half of all samples screened in HIV-1 integrase assays, the productivity by these organisms was limited. The reasons for this are not readily apparent, but could include weaker secondary metabolite potential of these organisms in terms of inhibition of HIV-1 integrase and less than ideal fermentation and media conditions to enhance secondary metabolite production. Only two classes of compounds were isolated from these sources and these are described below.

1. Hexacyclic polyketides

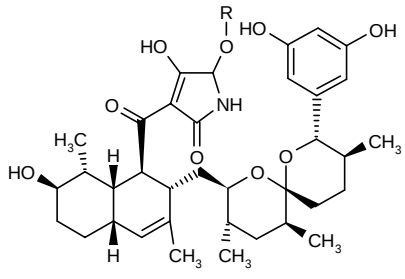
Integramycin

Integramycin (**106**), a hexacyclic polyketide consisting of a decalin, a bis-spiropyranyl, a 3,5-dihydroxyphenyl and a tetramic acid-type residue, was produced by an *Actinoplanes* sp. (48). This compound inhibited the coupled and strand transfer reactions with IC_{50} values of 3 and 4 μM, respectively. The methyl ether **107** displayed comparable activity (IC_{50} = 6.1 μM) in these assays. However, the mixture of epimeric pyridinium tetraacetates (**108**) was substantially less active (IC_{50} = 30-40 μM) in the strand transfer assay. Whether the loss of activity is due to the substitution of the hydroxy/methoxy group with the pyridinium group or concomitant acetylation of the phenolic/hydroxy groups is unclear.

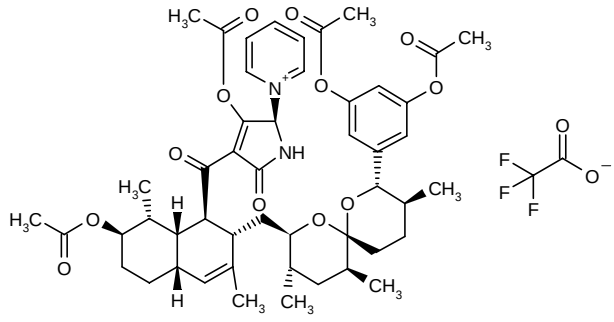
2. Nonribosomal bicyclo aromatic peptides

Complestatins and chloropeptin

Isocomplestatin (**109**), complestatin (**110**) and chloropeptin I (**111**), the latter a chemically rearranged product (49), are bicyclic hexapeptides. The only structural difference among these compounds is the connectivity of the indole ring of the tryptophan residue with the phenyl ring of the dihydroxyphenylalanine residue forming the same-size second macrocycle. Complestatins A (**112**) and B (**113**) are indole oxidized derivatives of isocomplestatin. Isocomplestatin and complestatins A and B were isolated from a *Streptomyces* sp. in an HIV-1 integrase screening program (50). Complestatin, an atropisomer of isocomplestatin, was originally isolated in 1980 and was re-isolated in 1994 together with chloropeptin I



(**106**) Integramycin: R = H
(**107**) R = CH_3



(**108**)

(51). It was reported to inhibit gp120-CD4 binding (IC_{50} = 1.3-2 μM) (51).

Isocomplestatin inhibited the coupled and strand transfer reactions with IC_{50} values of 0.2 and 4.0 μM, respectively. Chloropeptin I, complestatin A and complestatin B were slightly less active, inhibiting the coupled reaction with IC_{50} values of 0.4, 0.8 and 1.7 μM, respectively, and strand transfer with IC_{50} values of 5, 12.5 and 12.5 μM, respectively. The slight differences in the activities of these compounds were attributed to the expected deviation of the tryptophan-containing 16-membered ring of chloropeptin I and oxidation of the indole ring. The hydrolytic fragments **114** and **115** with intact macrocyclic rings retained almost all of the activities, exhibiting IC_{50} values of 0.6 and 0.3 μM and 8.0 and 6.0 μM, respectively, in the coupled and strand transfer assays (50, 52). However, opening of one of the macrocyclic rings resulted in a significant decrease in the integrase-inhibitory activity (see compounds **116-118**). Isocomplestatin was shown to inhibit preintegration complexes from HIV-1-infected cells with an IC_{50} value comparable to that for the full-length recombinant integrase enzyme.

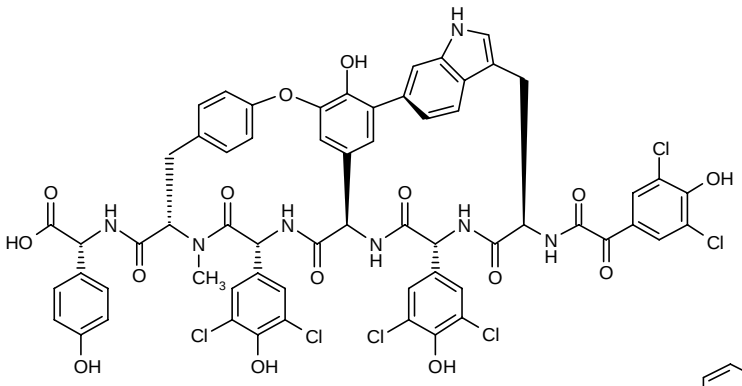
Isocomplestatin was equipotent in the 3'-end processing and strand transfer assays (IC_{50} = 0.2 μM), suggesting that it may preferentially bind to the uncomplexed enzyme, unlike the diketo acids which require binding to the viral DNA end (50). Unlike the diketo acids, isocomplestatin was shown to inhibit the HIV-1 disintegration activity of both the intact protein and the catalytic core domain of integrase (amino acids 50-212) with comparable potency (IC_{50} = 0.5 μM), indicating that it binds to the core domain of the integrase. Isocomplestatin also inhibited the integrase protein from several related retroviruses (*e.g.*, recombinant feline immunodeficiency virus [rFIV], IC_{50} = 0.5 μM; recombinant simian immunodeficiency virus [rSIV], IC_{50} = 0.1 μM).

Complestatin, chloropeptin I and the hydrolytic fragments **114** and **115** blocked viral replication in a multiple-cycle viral replication assay with IC_{95} values of 5, 5, 5 and 7.5 μM, respectively. The IC_{50} of isocomplestatin in the same assay was 0.2 μM. Complestatin and chloropeptin I also inhibited the binding of gp120 to CD4 with IC_{50} values of 2 and 1.3 μM, respectively (51). These compounds were further reported to form syncytia in cell culture (51). Similarly, we found that isocomplestatin also formed syncytia at micromolar levels in cell culture. Therefore, it is expected that inhibition of viral replication by isocomplestatin or complestatin is the result of the cumulative effect of their *in vitro* activities, including inhibition of HIV-1 integrase. Of all the natural products discovered, these bicyclo-hexapeptides displayed significant antiviral activity with a large therapeutic window.

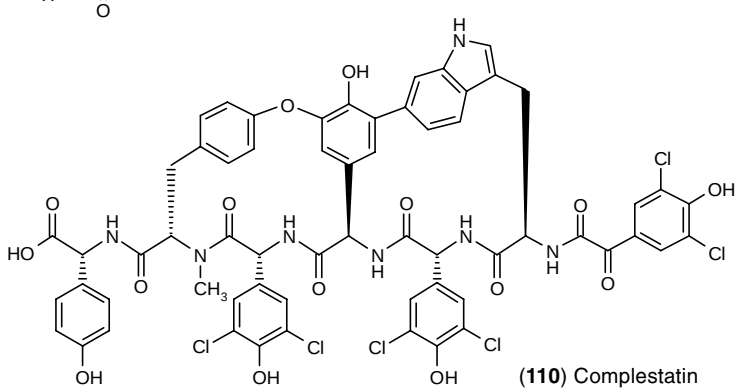
Dereplication

The repeated rediscovery of the same natural products in a random natural product screening effort can negatively impact the discovery process and hinder, if not completely prevent, any progress. Therefore, prior to embarking on a screening effort for natural products, all concerned must be aware of the issues that can and will arise during the exercise. Thus, an early, efficient and cost-effective targeted dereplication strategy to weed out all natural products that are already known to affect the biological processes of a particular assay/target is of paramount importance for overall success.

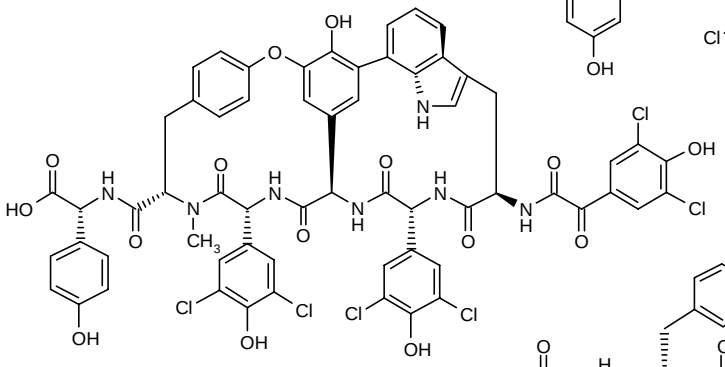
The term "dereplication" can mean different things to different people. We have defined dereplication as the identification of known compounds in a particular assay/target. Hence, dereplication, as described here, is a targeted dereplication against a list of previously identified HIV-1 integrase inhibitors. We ran all newly isolated inhibitors on a reversed-phase diode array HPLC system using a broad standard gradient to build up a library of retention times and ultraviolet spectra. This library was regularly updated with newly identified compounds. All active and prioritized extracts were first subjected to the HPLC-based targeted dereplication, and when a compound was found to match any of the known integrase inhibitors, its identity was confirmed by coinjection with an authentic sample of the known inhibitor and reconfirmed by LCMS. It was especially satisfying that even after screening 200,000 extracts spanning 5 years, very few compounds were rediscovered or re-isolated more than once. In fact, many of the inhibitors reported in this article



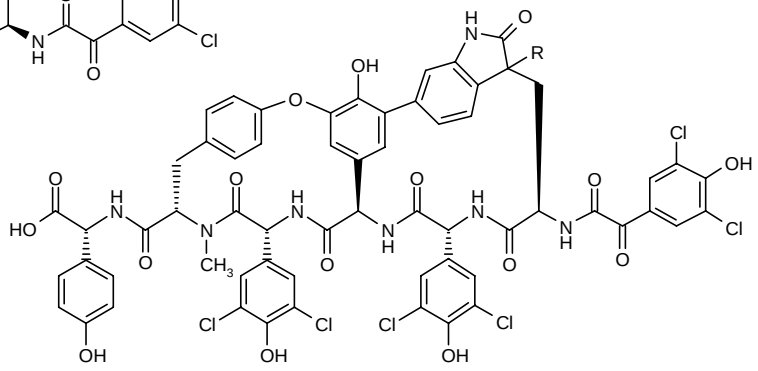
(**109**) Isocomplestatin



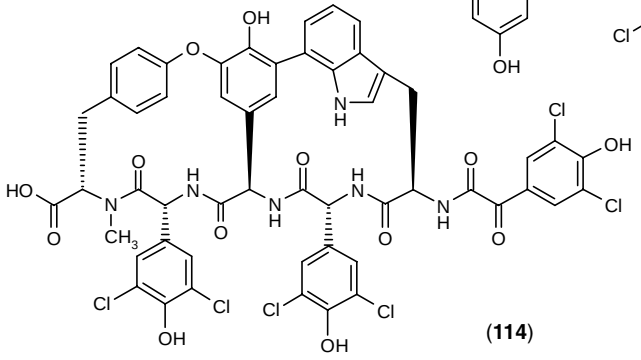
(**110**) Complestatin



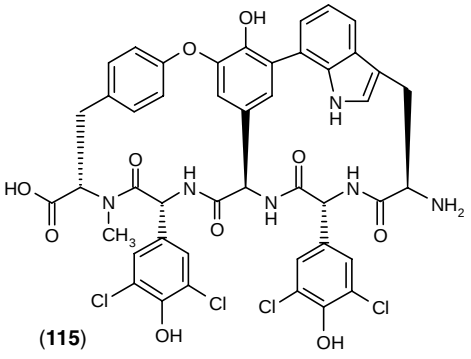
(**111**) Chloropeptin



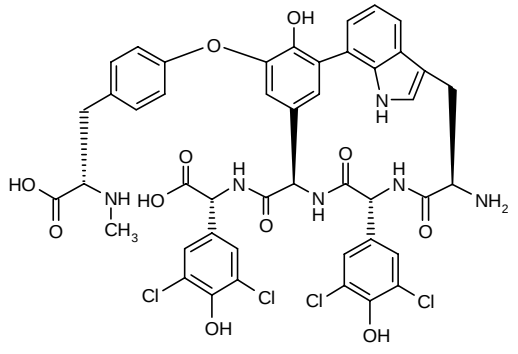
(**112**) Complestatin A: R = H
(**113**) Complestatin B: R = OH



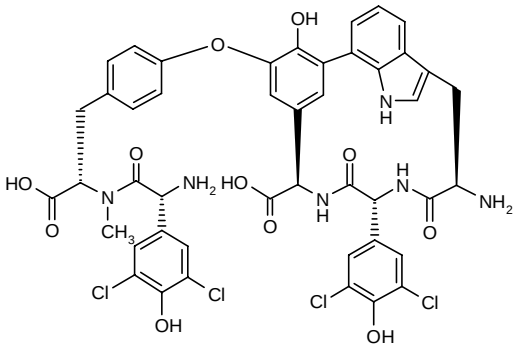
(**114**)



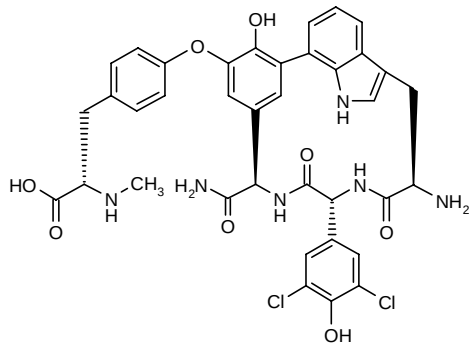
(**115**)



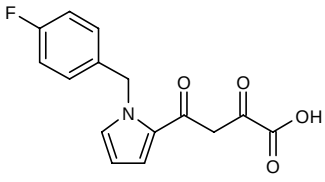
(**116**)



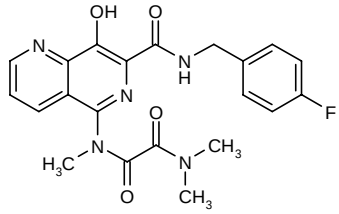
(**117**)



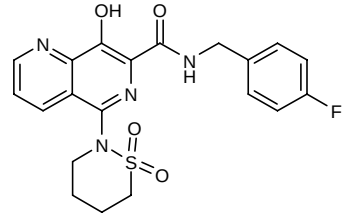
(**118**)



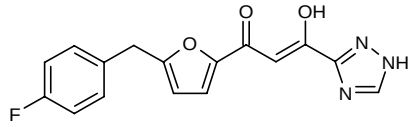
(**119**) L-731988



(**120**) L-870812



(**121**) L-870810



(**122**) S-1360

were discovered only once. Those found more than once include integric acid, equisetin, integracide A, isochaetochromin B_1, deoxyfunicone, hispidin and caffeic acid. However, none of these compounds was found more than 3 times and most of them were found only twice. The absence of the repeated discovery of known natural product HIV-1 integrase inhibitors indicates the diversity of the extracts, and thus of the microbial sources. However, the relative abundance of inhibitors in the extracts could also play a role in the detection of repeat inhibitors. In addition, the assay protocol was revised as we progressed, which may have contributed to the discovery of a diversity of structures. Had there been repeat inhibitors present in active extracts, we were uniquely positioned for an effective, rapid and quantitative dereplication of inhibitors at the earliest stage of discovery.

Key non-natural product developments leading to clinical candidates

While the natural product screening campaign was in progress, L-731988 (**119**), a diketo acid, was discovered at Merck by screening of a chemical collection and found to be a potent inhibitor of strand transfer activity (IC_{50} = 0.05 μM) with antiviral activity (CIC_{95} = 1 μM) (13). This lead was further optimized and resulted in a series of potent diketo acids with improved activity (53, 54). However, the strong electrophilic nature of the 1,3-diketone was undesirable and reduction of electrophilicity was required (54). Further rational optimization of the lead diketo acid class led to the naphthyridinecarboxamides L-870812 (**120**) and L-870810 (**121**). L-870812 inhibited HIV-1 and simian-human immunodeficiency virus (SHIV) with an IC_{50} value of 40 nM. This compound exhibited sustained suppression of viremia, preserved CD4 levels and permitted virus-specific cellular immunity in SHIV-infected rhesus macaques, and provided proof of concept that inhibition of HIV-1 integrase may be effective in controlling HIV in humans (55). As a result, a slightly superior analogue, L-870810, a potent inhibitor of strand transfer (IC_{50} = 8 nM) and 3'-end processing (IC_{50} = 85 nM) with potent antiviral activity (CIC_{95} = 15-100 nM), was selected for clinical development by Merck (54, 56). S-1360 (**122**; IC_{50} for strand transfer = 20 nM, CIC_{95} = 0.74 μM) is the second compound to be advanced into clinical development (54, 57) by Shionogi. The key pharmacophores of these compounds are the 1,3-diketo acid or the 3-hydroxyketone, which binds to two Mg^{2+} ions at the active site. The carboxyl group of the diketo acid contributes a lone pair and participates in the coordination of

one of the two Mg^{2+} ions with the diketo group, which coordinates the second Mg^{2+} ion at the active site. The Merck group cleverly replaced the carboxyl group with a nitrogen atom at equidistance, leading to naphthyridines that possess the potency and physical properties suitable for development as drugs, as exemplified by the clinical development of L-870810 (58). The β-hydroxyketo groups are also present in many of the natural products, which presents the opportunity for interaction with the metal-binding domain of the integrase enzyme, the same site where these key synthetic inhibitors bind and exert their antiviral activity.

Conclusions

In this review, we have discussed the discovery of 24 novel classes of chemically diverse natural product inhibitors of HIV-1 integrase resulting from over 5 years of screening of microbial extracts using freshly prepared extracts, as well as extracts stored in library formats, comprising over 200,000 crude extracts. These inhibitors represented a wide variety of natural product classes with molecular weights ranging from 180 to 1663 Da and IC_{50} values for HIV-1 integrase-inhibitory activity ranging from 50 nM to > 100 μM. The compound classes are broadly represented by phenolic and nonphenolic polyketides, isoprenoids and nonribosomal peptides. The nonaromatic polyketides are represented by equisetin, phomasetin, integric acid, cytosporic acid, coprophilin, integrasone and roselipins. Phenolic polyketides are represented by a vast majority of compounds containing more than one phenolic group, the most potent containing a catechol (*e.g.*, integrastatins, hispidin, caffeic acid) and/or beta-hydroxyketo (*e.g.*, xanthoviridicatins, isochaetochromins, terephenyllins) or resorcinilic acid (*e.g.*, integracins, exophillic acid, aquastatin, xerocomic acid) unit, and the mixed polyketide integramycin. The isoprenoids were represented by triterpenoids (*e.g.*, integracides), sesterterpenoids (*e.g.*, ophiobolins) and diterpenoids (*e.g.*, nalanthalide). The nonribosomal peptide inhibitors were represented by the linear hexadecapeptides (*e.g.*, integramides) and the bicyclo-hexapeptides (*e.g.*, isocomplestatin A and chloropeptin I). A number of these compounds were potent inhibitors of the strand transfer reaction of HIV-1 integrase. Isocomplestatin A and chloropeptin I were the best natural product inhibitors discovered in our program, showing potent *in vitro* and cell-based activities.

While there was some chemical modification effort on integric acid and integracide, none of the natural products or derivatives were further developed. However, the early discovery of a number of these compounds (*e.g.*, equisetin, integric acid, integramycin and integracide A) led to further mechanistic understanding of HIV-1 integrase and the refinement of *in vitro* and cell-based assays, allowing the discovery of lead synthetic diketo acid inhibitors, which upon further optimization resulted in several clinical candidates (L-870810, *etc.*). The β-hydroxyketo group has been recognized as a key structural requirement of the diketo acids and naphthyridinecarboxamides, which interacts with the bivalent cation (Mg^{2+})-binding domain of the enzyme (reviewed in 54). β-Hydroxyketo groups are also present in many of the natural products. Although we failed to discern the impact of this group on activity due to low potency, it is not clear whether this would have helped to develop any of these compounds into formal leads.

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