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Library-Based Discovery and Characterization of Daphnane Diterpenes as Potent and Selective HIV Inhibitors in *Daphne gnidium*

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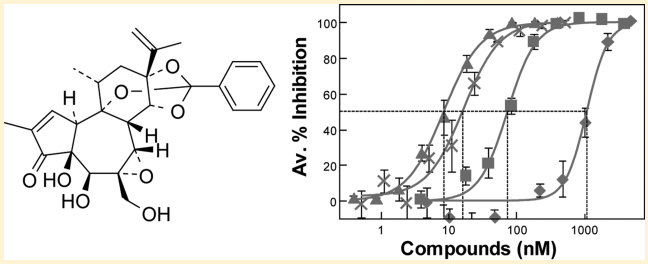
Supporting Information

ABSTRACT: Despite the existence of an extended armamentarium of effective synthetic drugs to treat HIV, there is a continuing need for new potent and affordable drugs. Given the successful history of natural product based drug discovery, a library of close to one thousand plant and fungal extracts was screened for antiretroviral activity. A dichloromethane extract of the aerial parts of *Daphne gnidium* exhibited strong antiretroviral activity and absence of cytotoxicity. With the aid of HPLC-based activity profiling, the antiviral activity could be tracked to four daphnane derivatives, namely, daphnetoxin (**1**), gnidicin (**2**), gniditrin (**3**), and excoecariatoxin (**4**). Detailed anti-HIV profiling revealed that the pure compounds were active against multidrug-resistant viruses irrespective of their cellular tropism. Mode of action studies that narrowed the site of activity to viral entry events suggested a direct interference with the expression of the two main HIV co-receptors, CCR5 and CXCR4, at the cell surface by daphnetoxin (**1**).



During the last two decades antiretroviral research has yielded a number of potent new drugs targeting various steps in the HIV life cycle. As a direct consequence, their implementation and use as part of regimens of highly active antiretroviral therapy (HAART) has in many countries led to a remarkable reduction of disease progression, improved quality of life, and increased life expectancy.[1,2] Nevertheless, even these outstanding global efforts toward therapy improvement have been unable to eliminate HIV in a patient after infection or to prevent the emergence of resistant viruses.[3,4] Consequently, and due to a continuing lack of accessibility of HAART in many countries and to high costs and logistic hurdles, HIV continues to spread through our populations at an alarming rate in resource-poor settings. Therefore, the development of new anti-HIV compounds is needed urgently to overcome the drawbacks of many current drugs, such as the expense, limitations in activity due to viral resistance, and their side effects.

Historically, natural products have been the most successful source of inspiration for development of new drugs.[5] In the field of antiretroviral drug discovery and development, compounds with new mechanisms of action, such as prostratin, calanolide A, and integric acid, have been discovered.[6−8] A betulinic acid derivative, dimethylsuccinylbetulinic acid (PA-457), is the first maturation inhibitor that has reached phase II clinical trials.[9]

The present study set out to identify new natural sources for HIV inhibitors and to isolate the active compounds. Screening of a library of more than 800 plant and fungal extracts for selective antiretroviral activity revealed that a CH_2Cl_2 extract from the aerial parts of *Daphne gnidium* L. (Thymelaeaceae) was by far the most potent sample. *D. gnidium* is a small evergreen shrub that is distributed widely in the Mediterranean basin. We report on the isolation and chemical characterization of the active constituents, the biological characterization of their antiretroviral constituents, and experiments to identify an underlying mechanism of action.

RESULTS AND DISCUSSION

A library of more than 800 plant and fungal extracts was screened at a final concentration of 10 μg/mL in a cell-based assay for activity to block the virus life cycle. The most active sample in this screening was a CH_2Cl_2 extract of the aerial parts of *D. gnidium*, which led to a 50% decrease in viral replication

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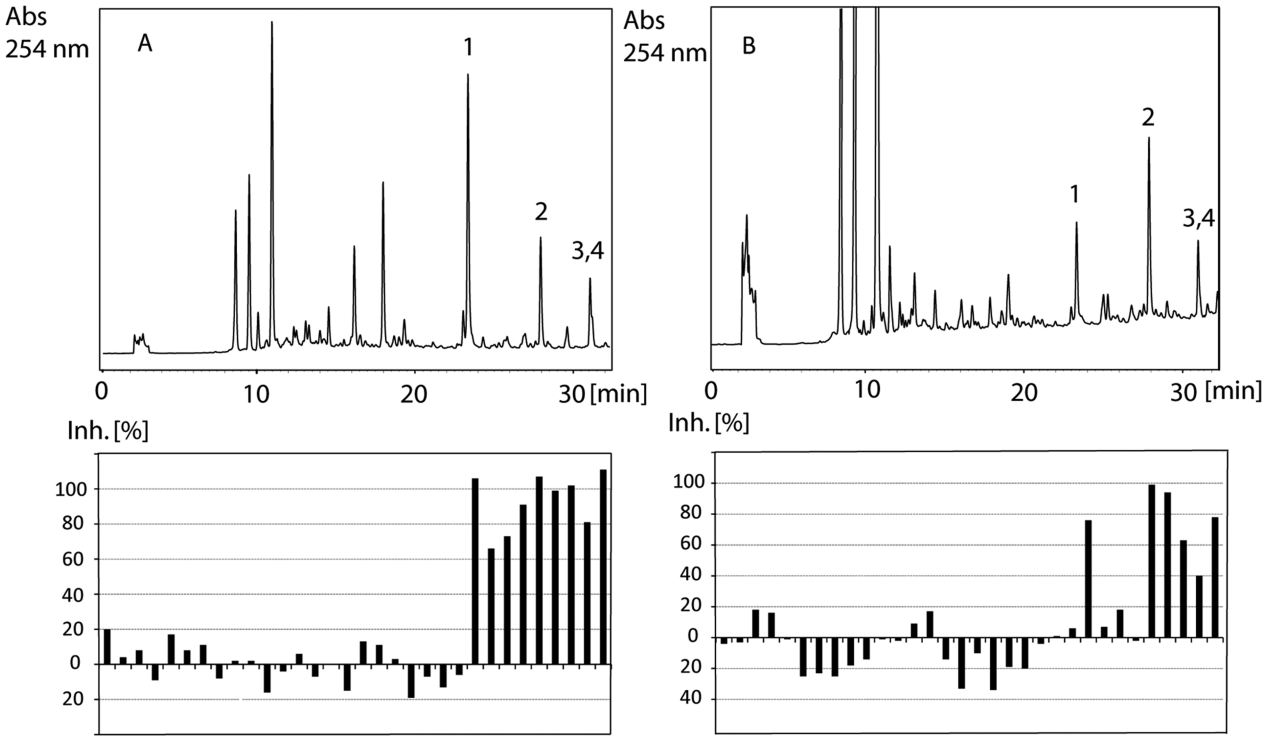

Figure 1. HPLC-based activity profiling. HPLC chromatograms of the CH_2Cl_2 extracts of the stems (top left) and leaves (top right) of *D. gnidium* and the corresponding activity profiles (% inhibition of cytopathic effect) of microfractions (below). SunFire C_{18} column, 30 μL (300 μg) injected. A gradient of 5−100% MeCN(aq) containing 0.1% formic acid in H_2O in 30 min was used. Fractions were collected at 1 min intervals, with detection at 254 nm. Peaks in the HPLC chromatograms are assigned to the isolated compounds **1**−**4**.

(EC_{50}) at around 280 ng/mL. Of note, this extract was equipotent against HIV-1 isolates with either one of the two main tropisms, i.e., viruses that use as co-receptor for cell entry either the chemokine receptor CXCR4 or CCR5. At the same time, the extract did not display any cytotoxicity in the human host cells at concentrations up to 50 μg/mL. On the basis of these positive attributes, it was decided to identify the active principle(s) from *D. gnidium.*

For this purpose, HPLC-based activity profiling was used, which combines the separation of a complex mixture with physicochemical data recorded online and with biological information obtained in parallel from time-based microfractionation of the HPLC effluent and subsequent bioassay.[10] The leaves and stems of *D. gnidium* were extracted separately in CH_2Cl_2. Small aliquots of the extracts (300 μg) were separated by HPLC, and fractions were collected at 1 min intervals in 96-deep-well microtiter plates, vacuum-dried, and then tested for antiviral activity at a 1/400 final dilution of the 10 μL fractions. As shown in Figure 1, the activity was found in both extracts in the late-eluting fractions (24−30 min) and matched well with three main UV absorbing peaks in the HPLC chromatograms. Since the concentration of these compounds appeared to be higher in the stem extract, this was selected for further investigation. To obtain sufficient amounts of material for biological and physicochemical characterization of the active substances, the purification scheme was scaled up. Separation of the CH_2Cl_2 extract of the stems by semipreparative HPLC afforded compounds **1**−**4**. They were identified using a combination of spectroscopic methods including ESIMS, 1D- and 2D-NMR spectroscopy, and comparison with literature data as a series of daphnane diterpenes, namely, daphnetoxin (**1**),[11] gnidicin (**2**),[12,13] gniditrin (**3**),[12,13] and excoecariatoxin (**4**).[14] The ^{1}H NMR data of compounds **1**−**4** and the ^{13}C NMR data of compounds **1** and **2** are provided as Supporting Information (Tables S1 and S2). The positive specific rotation value of **1** ($[\alpha]_D^{20}$ +45 (*c* 0.2, $CHCl_3$); $[\alpha]_D$ +63,[11] $[\alpha]_D^{25}$ +35 (*c* 0.06 $CHCl_3$)[15]) confirmed the absolute configuration to be identical to that reported for daphnetoxin.

The four pure compounds were next tested for their antiviral activity against two prototypic reference strains of HIV-1 differing in their co-receptor usage. As shown in Figure 2, these compounds inhibited equally well the replication of both CXCR4- and CCR5-tropic HIV-1. However, the compounds differed in their potency, with daphnetoxin (**1**) and gnidicin (**2**) being the most potent, and excoecariatoxin (**4**) the least active compound for both viruses (Figure 2). Importantly, none of the purified compounds displayed any cytotoxic activity at the doses assayed, thus yielding a high selectivity index in each case. Based on structural similarities of the isolated daphnanes and their varying concentrations in the extract, further investigations were focused on the most abundant compound, daphnetoxin (**1**).

A critical issue in the long-term clinical management of HIV disease is the development of drug resistance. To address the mechanism of action and potency of this possibly new class of inhibitors, **1** was assayed against a panel of viruses possessing mutations that confer selective resistance either to several nucleosidic reverse transcriptase inhibitors or to various protease inhibitors. As shown in Table 1, virus 695-RT was demonstrated to be highly resistant to nucleoside analogues and to non-nucleoside inhibitors, with resistance factors ranging from 11 to >300 depending on the drug considered. Similarly, the multiply mutated clonal virus 106-PR renders all current protease inhibitors quite ineffective. Daphnetoxin (**1**) inhibited

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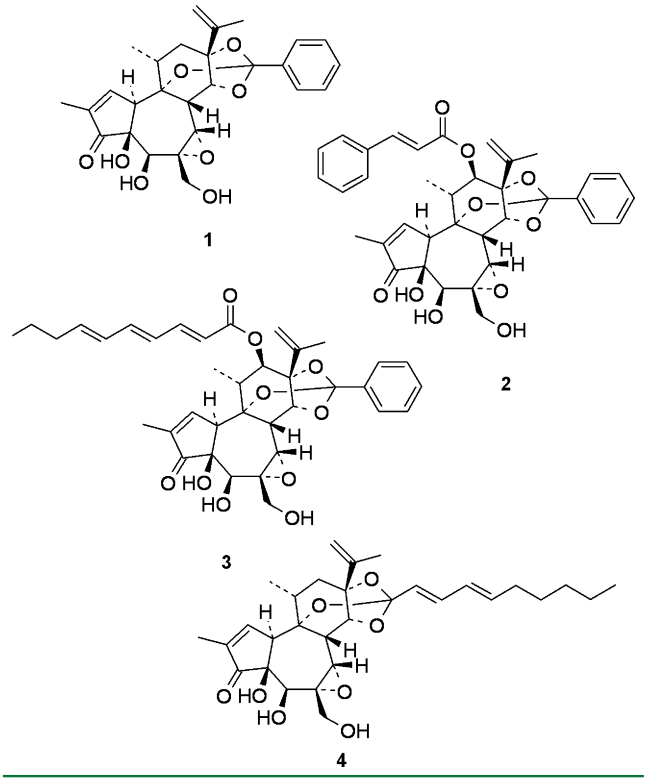

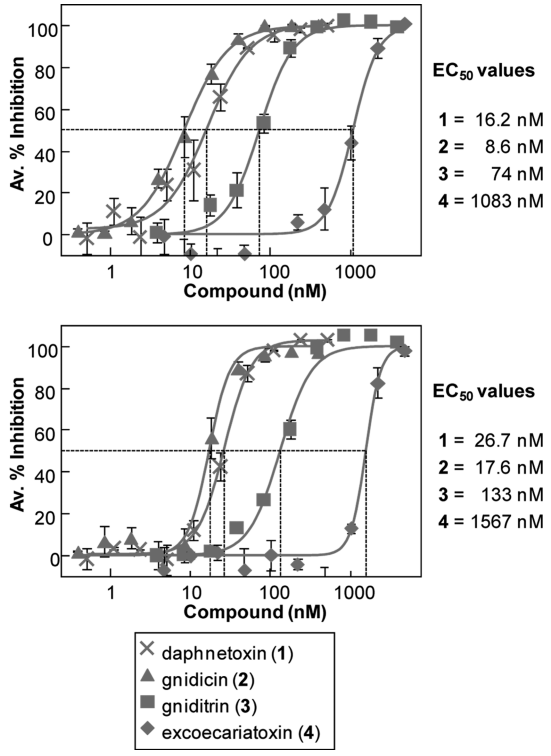

Figure 2. Antiviral activity profiles of **1**–**4**. Inhibition of HIV replication by each compound was assessed using the CXCR4-tropic HIV-1 strain NL4-3 (top graph) or the CCR5-tropic HIV-1 strain NL-AD87 (bottom graph). Each data point represents the mean ± standard deviation.

the replication of both mutant viruses with similar efficacy to prototypic wild-type HIV-1.

A determination of the mode of action of daphnetoxin (**1**) was then conducted. Related diterpenoid compounds have been described in the literature as being able to block HIV entry into cells.[16−19] To demonstrate specifically that a similar impairment occurs with daphnanes, two types of pseudotyped HIV particles were generated that contained a genetically identical viral core. One type expressed at its surface the genuine HIV-1 gp160-derived envelope, while the other expressed that of the vesicular stomatitis virus (VSV). Of note, compared to HIV, VSV relies on a different and unrelated mechanism for cell entry. Therefore, if the hypothesis made was correct, then infection by VSV-pseudotyped particles would not be blocked by daphnetoxin (**1**). As shown in Figure 3, the single-cycle infection triggered by both pseudoparticles was blocked efficiently by the reverse transcriptase inhibitor efavirenz, while in the presence of the HIV envelope-specific fusion inhibitor T20, only the function of HIV-pseudotyped particles was inhibited. In contrast, VSV-pseudotyped particles were fully competent to complete the infection cycle, yielding 100% of signal. A dose-dependent inhibition profile was observed when HIV-pseudotyped particles were challenged with **1**. No inhibitory effect on the infectivity of VSV-pseudotyped particles was observed, thereby demonstrating that HIV replication was inhibited via blocked virus entry.

It was next investigated whether this inhibition could be attributed to a down-regulation of the surface expression of the chemokine receptors CXCR4 and CCR5 serving as HIV co-receptors. This has been described for other diterpenes,[18,19] and we sought to assess this possibility for daphnetoxin (**1**) in a situation closer to a clinical context, i.e., on primary human blood cells. Human peripheral monocytes were obtained from a healthy donor and cultured for 48 h in vitro in the presence of PHA and IL-2. Thereafter, activated PBMCs were incubated in the presence of 20 nM **1** or vehicle, and cells were analyzed directly at different time points by flow cytometry for their cell-surface expression of CXCR4 and CCR5. As a control for possible nonselective down-regulation of surface membrane receptors, the expression of CD69 was also measured under the same conditions. Results are depicted in Figure 4 as percentage of receptor mean expression as a function of time after treatment with **1**. Following addition of this compound, the expression of both HIV co-receptors CXCR4 and CCR5 declined rapidly to a minimal level. In contrast, the expression of CD69 slightly increased during the same period. Of note, the expression of HIV co-receptors remained suppressed 48 h after initiation of treatment with **1**.

Daphnane diterpenes are characteristic constituents of the family Thymelaeaceae. Compounds **1**–**4** have been reported from various *Daphne* species.[20,21] However, only daphnetoxin

Table 1. Inhibition of Multiresistant HIV Strains

virus	compound	EC_{50} (nM)	Rf[a]
695-RT	AZT[b]	200	11
	3TC[b]	>20 000	>300
	efavirenz[b]	889	222
	1	21	1
	saquinavir[c]	12	1
106-PR	lopinavir[c]	160	10
	darunavir[c]	13 660	>300
	atazanavir[c]	324	7
	1	22	1
	AZT[b]	6	1

[a]Rf: resistance factor, ratio of EC_{50} for mutant versus wild-type virus. [b]Reverse transcriptase inhibitor. [c]Protease inhibitor.

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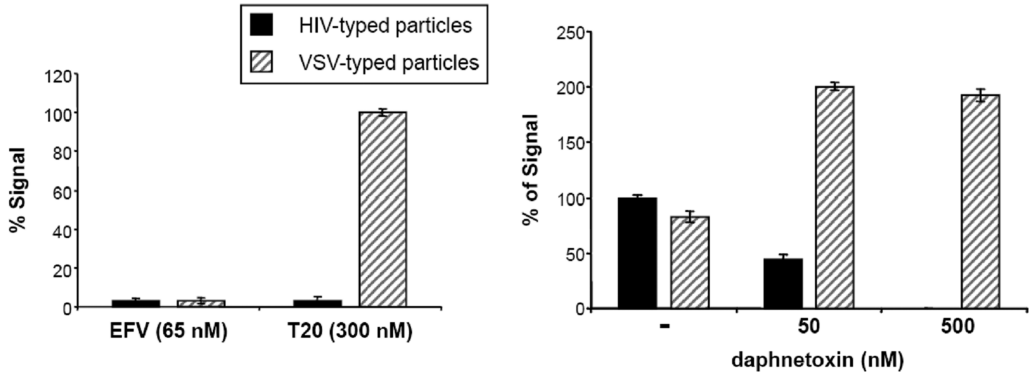

Figure 3. Assessment of the mode of action of daphnetoxin (**1**) probing the HIV-1 envelope. HIV-pseudotyped particles using HIV-1 Env in trans (black bars) and VSV-pseudotyped particles carrying the VSV envelope (hatched bars) were used to transduce the reporter cell line. Throughout the cultivation period, reporter cells were treated either with the non-nucleosidic reverse transcriptase inhibitor efavirenz or with the HIV entry inhibitor T20, or, in a parallel experiment, infected cells were treated or not with increasing doses of **1** at the indicated concentrations. Results are expressed as means ± standard deviation.

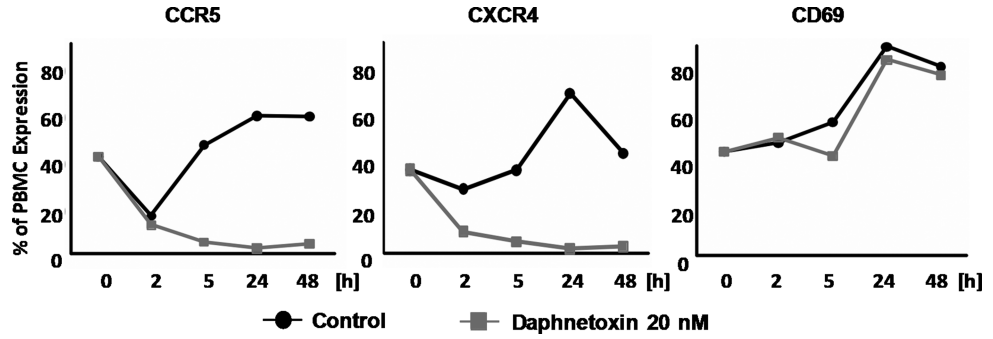

Figure 4. Daphnetoxin (**1**) down-regulates the HIV receptors CXCR4 and CCR5 in PBMCs. After activation of PBMCs with PHA and IL-2 for 48 h, cells were treated with **1** at 20 nM. Expression of receptors was determined by using specific antibodies at different time points along drug treatment (0, 2, 5, 24, and 48 h). Results are shown as percentage of cell expression of each receptor. PBMCs were analyzed using forward versus side scatter dot plots and cytometer fluorescence histograms.

(**1**) has been previously isolated from *D. gnidium*,[22] and compounds **2**–**4** are, to our knowledge, described here for the first time from this species.

When the present study was initiated, there was no previous knowledge about the antiretroviral potential of daphnane-type diterpenoids. While the study was in progress, antiviral daphnanes were reported from *Trigonostemon thyrsoideus* (Euphorbiaceae).[23,24] One of the compounds, trigonothyrin F, prevented the cytopathic effects of HIV-1 IIIB in susceptible cells at sub-μM concentrations. However, the other diterpenes were significantly less potent, and the mode of action was not studied.

It is demonstrated here that daphnanes are active against both CXCR4- and CCR5-tropic viruses and provide evidence that daphnetoxin (**1**) abrogates the surface expression of the two chemokine receptors CXCR4 and CCR5 on human primary mononuclear cells. However, treatment with this compound did not lead to a general shut-off of membrane protein expression, since CD69 expression was not perturbed by the presence of **1**. Interestingly, daphnanes such as daphnetoxin (**1**) have been shown to activate the classical (α and β) and novel (δ) members of the protein kinase C family.[22,25] In more general terms, diterpenes with structurally related scaffolds such as deoxyphorbol and jatrophane esters have been previously found to possess potent antiretroviral activity. The best studied compound is prostratin, a non-tumor-promoting deoxyphorbol from the Samoan traditional plant *Homalanthus nutans*.[6,26] This compound possesses a dual mode of action, through PKC-dependent NF-κB activation of latent HIV,[27] and in turn, activation of these isoforms leads to a down-regulation of the surface expression of CXCR4 and CCR5 not only on PBMCs but also on various cell lines.[16−18] Therefore, the present results are in line with those reported in the literature, and it can be surmised that the four daphnanes from *D. gnidium* exert their anti-HIV activity at least in part via the modulation of members of the PKC family, leading to the down-regulation of the surface expression of both HIV co-receptors.

However, although cell-surface labeling experiments provide evidence that daphnetoxin (**1**) abrogates the surface expression of these two key cell-surface cofactors for HIV-1 entry, it still remains possible that this class of inhibitor may simultaneously or even predominantly exert additional anti-HIV effects at distinct later steps of the virus life cycle. Pseudotyped HIV constructs were then employed for discriminating effects on viral entry from other modes. The otherwise isogenic nature of the virus constructs carrying different envelopes ensures by strict functional separation that no other viral functions such as structural components mediated by Gag or the enzymatic functions (reverse transcriptase, RNase H, integrase, or protease) would contribute in an uncontrolled way to the antiviral activity of any compound.

Multiple reports have validated the suitability of pseudotyped viruses for functional interrogation of viral envelope func-

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tion.[28,29] As at the active concentration of **1**, no significant inhibitory effect was observed with VSV-pseudotyped HIV, it was concluded that a putative intracellular effect, for example on kinases or elements of signaling pathways that could drive alternative mechanisms of action, would be secondary. Moreover, for HIV variants that carry mutations conferring broad-class resistance for inhibitors of HIV protease or polymerase, no difference in the inhibitory activity of **1** was noted. This suggests that the enzymatic functions of HIV are not targeted by daphnetoxin (**1**).

Given the limited data, structure−activity considerations remain highly preliminary. If one takes also trigonothyrin F[24] into account, a phenyl moiety at C-1′ (**1**−**3**, trigonothyrin F) seems essential for sub-μM activity. On the other hand, the role of substituents at C-12 and C-13 seems less clear, although a fatty acid ester moiety at C-12 appears unfavorable. Some modifications at rings A and B appear less critical for activity, if one compares **1** and trigonothyrin F. With respect to lipophilicity no obvious pattern could be seen between active and less active daphnanes (cLogP: daphnetoxin (**1**) 0.69, gnidicin (**2**) 2.45, gniditrin (**3**) 3.59, excoecariotoxin (**4**) 3.57, trigonothyrin F 4.19). We thus believe that the differences in activity result from the differing abilities of these daphnanes to activate PKC-dependent pathways,[16,17,22,30] but further investigations are needed to substantiate structure−activity relationship of daphnanes and their patterns of PKC activation.

From a more general perspective, this study demonstrates the potential of HPLC-based activity profiling as an efficient strategy for the identification of antiviral compounds and their targeted isolation from complex plant extracts.

EXPERIMENTAL SECTION

General Experimental Procedures. Semipreparative HPLC was performed on an Agilent 1100 system equipped with a PDA detector. Separations were performed on a SunFire C_{18} column (150 × 10 mm i.d, 5 μm, Waters) equipped with a precolumn (10 × 10 mm). The flow rate was 4 mL/min, and detection was at 243 nm. HPLC-based activity profiling and final purification of **3** and **4** were performed on a Waters 2695 Alliance system equipped with a 996 PDA detector and a C_{18} SunFire column (150 × 3 mm, 3.5 μm, Waters) connected to a precolumn (10 × 3 mm). The flow rate was 0.4 mL/min. Optical rotation was measured on a Perkin-Elmer model 341 polarimeter. NMR spectra were recorded on a Avance III spectrometer (Bruker BioSpin) equipped with a 5 mm BBO probe (^{13}C NMR) or a 1 mm TXI microprobe (^{1}H and 2D-NMR), at 500 (^{1}H) and 125 MHz (^{13}C). ESIMS were obtained on a Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics).

Biological Material. The screening library consisted of plant and fungal extracts archived as 10 mg/mL solutions in DMSO. Stems and leaves of *Daphne gnidium* were collected in July 2006 near San Pantaleo (Sardinia, Italy). The plant material was identified by one of the authors (M.H.). A voucher specimen (Nr. 333) is kept at the Division of Pharmaceutical Biology, University of Basel. The leaves and stems were separated from each other prior to extraction.

Extraction and Isolation. The powdered, dried stems (57 g) and leaves (171 g) were percolated separately with CH_2Cl_2 (1.1 L) to provide after evaporation to dryness under reduced pressure 911 mg and 12.4 g of the stem and leaf extracts, respectively. To localize the bioactivity, both extracts were separated on a C_{18} analytical HPLC column. Volumes of 30 μL corresponding to 300 μg of extract were injected. A gradient of 5−100% MeCN(aq), containing 0.1% formic acid in water in 30 min, was used. Fractions were collected at 1 min intervals into a 96-deep-well plate, evaporated to dryness, and redissolved in DMSO (10 μL) for assessment of their antiviral activity.

The CH_2Cl_2 extract of the stems was chosen for preparative isolation. Separation by repeated injections on a semipreparative HPLC column (60−100% MeOH in 30 min) yielded pure **1** (10.4 mg) and **2** (4.2 mg), as well as crude **3** (3 mg) and **4** (3.2 mg). Final purification of the latter compounds by repeated injections on an analytical HPLC column gave pure **3** (1.3 mg) (80−100% MeOH in 30 min) and **4** (0.65 mg) (55−80% MeOH in 35 min). The purity was estimated to be approximately 95% for daphnetoxin (**1**) and over 90% for compounds **2**−**4** from ^{1}H NMR spectra.

Cell Culture. The reporter cell line HeLa-SxR5 stably expresses the CD4 receptor, the CCR5 chemokine receptor, and the HIV-1 long terminal repeat fused upstream of the bacterial reporter gene LacZ coding for β-galactosidase. Of note, HeLa cells constitutively express the CXCR4 chemokine receptor. In these reporter cells, the activity of β-galactosidase is proportional to the extent of viral replication. HeLa-SxR5 and human embryonic kidney cells (HEK293T) were maintained in high-glucose DMEM medium supplemented with 10% fetal bovine serum and cultivated at 37 °C in a 7% CO_2 atmosphere.

HIV Replication Assay. HEK293T cells were transfected with wild-type proviruses NL4-3 (CXCR4-tropic) or NL-AD87 (CCR5-tropic) or with proviruses encoding mutations that confer multi-resistance either to protease inhibitors (virus 106-PR) or to reverse transcriptase inhibitors (virus 695-RT). For all transfection experiments Lipofectamine2000 (Invitrogen) was used as lipofectant. Following transfection, HEK293T cells were dispensed into 96-well plates and incubated in the presence of HeLa-SxR5 reporter cells and test substances for 4 days. At the end of the incubation period, plates were processed for determination of β-galactosidase activity. Colorimetric readout (405 nm) was transformed into percent viral inhibition following normalization of data with positive and negative control wells included in each 96-well plate.[31,32] When appropriate, the effect of drugs on HIV replication was modeled using dose−response curve-fitting software (XLfit v4.0.1, IDbusiness solutions, Guilford, UK), and the effective concentration 50 (EC_{50}), i.e., the dose of compound leading to a 50% decrease in viral replication, was deduced from the fitted curves.

Pseudotyping Assay. To generate pseudotyped particles, HEK293T cells were cotransfected with a NL4-3-derived provirus coding for a defective envelope and with a plasmid expressing either the full-length HIV envelope from NL4-3 virus or the envelope of the vesicular stomatitis virus. Two days post-transfection, cell culture supernatants containing pseudotyped particles were harvested, centrifuged at low speed to remove debris, and used to transduce HeLa-SxR5 in the presence or absence of relevant compounds (Figure 3). Three days later, β-galactosidase activity was quantitatively determined as described above.

Cytotoxicity. For the assessment of cytotoxicity the same cell culture conditions as used for anti-HIV activity determination were utilized with the exception that no proviral DNA was transfected. After a 4-day incubation period, cytotoxicity was determined using AlamarBlue reagent as per manufacturer's instructions (Invitrogen).

Preparation of Blood Mononuclear Cells (PBMCs). PBMCs from a healthy donor were obtained via cell preparation tubes (CPT, 4 mL, BD Biosciences). After centrifugation, PBMCs were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI supplemented with 10% of fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. PBMCs were seeded in a 24-well cell culture plate at a density of 1×10^6 cells/mL. PBMCs were activated with 2.5 μg/mL of phytohemagglutinin (PHA, Sigma-Aldrich) and 300 IU/mL of interleukin-2 (IL-2, Sigma-Aldrich) for 48 h.

CXCR4, CCR5, and CD69 Detection by Flow Cytometry. Activated PBMCs were treated for 48 h with daphnetoxin (**1**) at a concentration of 20 nM or with vehicle (0.25% v/v DMSO in complete RPMI culture medium supplemented with 100 IU/mL of IL2). At different time points, T0, T2h, T5h, T24h, and T48h, 100 μL of cell suspension was transferred to a FACS tube and washed with PBS. Cells were incubated with FITC-coupled mouse anti-human CXCR4 or CCR5 (R&D Systems) or with PE-coupled mouse anti-human CD69 (R&D Systems) or with the appropriate mouse IgG1 isotype control monoclonal antibody (R&D Systems) for 40 min at 4 °C in the dark. After washing in PBS, cells were resuspended in 200 μL

of CellFix solution (BD Biosciences) and analyzed by a FACSCantoII flow cytometer.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR data of compounds **1**−**4**, ^{13}C NMR data of compounds **1** and **2**, and ^{1}H NMR spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

Dedicated to Dr. Gordon M. Cragg, formerly of the National Cancer Institute, Frederick, MD, for his pioneering work on the development of natural product anticancer agents.

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