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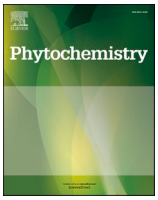

Design, synthesis and screening of a drug discovery library based on an *Eremophila*-derived serrulatane scaffold

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ABSTRACT

Chemical studies of the aerial parts of the Australian desert plant *Eremophila microtheca* afforded the targeted and known diterpenoid scaffolds, 3,7,8-trihydroxyserrulat-14-en-19-oic acid and 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid. The most abundant serrulatane scaffold was converted to the poly-methyl derivatives, 3-hydroxy-7,8-dimethoxyserrulat-14-en-19-oic acid methyl ester and 3,7,8-trimethoxyserrulat-14-en-19-oic acid methyl ester using simple and rapid methylation conditions consisting of DMSO, NaOH and MeI at room temperature. Subsequently a 12-membered amide library was synthesised by reacting the methylated scaffolds with a diverse series of commercial primary amines. The chemical structures of the 12 undescribed semi-synthetic analogues were fully characterised following 1D/2D NMR, MS, UV, ECD and $[\alpha]_D$ data analyses. All compounds were evaluated for their anthelmintic, anti-microbial and anti-viral activities. While none of the compounds significantly inhibited motility or development of the exsheathed third-stage larvae (xL3s) of a pathogenic ruminant parasite, *Haemonchus contortus*, the tri-methylated analogue induced a skinny phenotype in fourth-stage larvae (L4s) after seven days of treatment (IC_{50} = 14 μM). Anti-bacterial and anti-fungal activities were not observed at concentrations up to 20 μM. Activity against HIV latency reversal was tested in inducible, chronically-infected cells, with the tri-methylated analogue being the most active (EC_{50} = 38 μM).

1. Introduction

Approximately 40% of the developed therapeutic drugs approved by the Food and Drug Administration (FDA) are natural products (NPs), NP derivatives, or NP-related synthetic mimetics (Newman and Cragg, 2020). A frequent strategy in NP drug discovery is the medicinal chemistry optimisation of a purified natural scaffold, which can generate unique screening libraries based upon the core scaffold (Barnes et al., 2016). Scaffolds in this context are three-dimensional structural elements found in nature that contain mono- or multi-functional groups, which can be used as chemical handles for semi-synthesis. Noteworthy examples of this semi-synthetic approach include libraries based on fredericamycin A (Abel et al., 2006; Pandey et al., 1981), manzamine A (Peng et al., 2010; Sakai et al., 1986), lipopeptide FR901379 (Iwamoto et al., 1994; Tomishima et al., 2008), leubethanol (Escarcena et al., 2015; Molina-Salinas et al., 2011; Perez-Meseguer et al., 2012), rosmarinic acid (Cardullo et al., 2019) and maslinic acid (Juan et al., 2006; Nejma et al., 2018).

Our approach for the generation of unique and rare compound libraries for drug discovery and/or chemical biology research is based on the use of purified NPs from Australian biota such as microfungi (e.g. phenylacetic acid library) (Kumar et al., 2015), mushrooms (e.g. tetrahydroanthraquinone library) (Choomuenwai et al., 2012), marine sponges (e.g. psammaplysin F) (Kumar et al., 2020) and plants (e.g.

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muurolane library) (Barnes et al., 2012). The abundant serrulatane diterpenoids isolated from the endemic Australian desert plant *Eremophila microtheca* (F. Muell. ex Benth.) F. Muell. have been an ongoing focus of our group (Barnes et al., 2013; Kumar et al., 2017). In the first report of previously undescribed NPs isolated from the aerial parts and roots of *E. microtheca* (Barnes et al., 2013; Kumar et al., 2017, 2018), the abundant NP 3,7,8-trihydroxyserrulat-14-ene-19-oic acid (**1**) was identified as an attractive scaffold for structural modification through a one-pot process where pivalic anhydride was prepared *in situ* as an intermediate and subsequently reacted with a small library of commercially available amines. However, the yield of this amide library was low (<19%) and all of the 10-membered library analogues displayed more than one Lipinski's "Rule of Five" violation (Kumar et al., 2017; Lipinski et al., 1997), which made them less attractive for NP drug discovery. Limited raw plant material (<20 g) for the earlier studies prevented more detailed semi-synthetic work.

However, with the supply of raw plant material (>10 kg) resolved due to several *E. microtheca* crops now established in and around Brisbane (Queensland, Australia), we re-investigated the semi-synthetic serrulatane chemistry with the goals of improving yields of the targeted amide compounds and making the library more drug-like by emphasising key physicochemical parameters for oral bioavailability, as detailed by Lipinski's "Rule of Five" (Lipinski et al., 1997). Herein we report on the design, synthesis and anti-infective screening of a discovery library based on the *Eremophila*-derived serrulatane scaffold, 3,7,8-trihydroxyserrulat-14-ene-19 oic acid (**1**). All previously undescribed semi-synthetic compounds were characterised using spectroscopic and spectrometric techniques. These compounds were further evaluated for anthelmintic activity using the pathogenic ruminant parasite *Haemonchus contortus*, anti-microbial activity against a panel of bacteria and yeast pathogens, anti-HIV activity using a cell-line model of inducible and chronic infection, and cytotoxicity.

2. Results and discussion

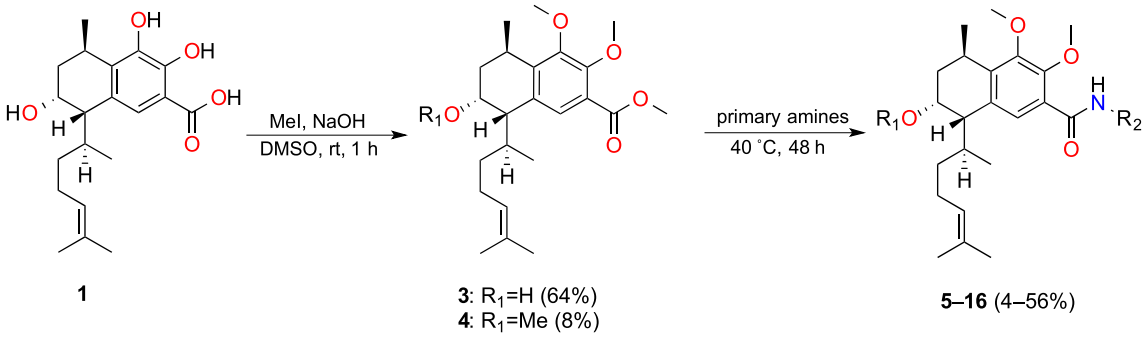
Large-scale CH_2Cl_2 extraction of the air-dried and ground aerial parts of *E. microtheca* followed by C_{18}-bonded silica flash column chromatography (MeOH/H_2O/0.1% TFA) and semi-preparative HPLC (H_2O/MeOH/0.1% TFA) afforded the previously reported and targeted compounds 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**1**, 625.5 mg) and 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**2**, 52.3 mg), respectively. Comparison of NMR, $[\alpha]_D$ and MS data with literature values confirmed these known compounds had been successfully purified (Barnes et al., 2013).

The most abundant serrulatane **1** had been previously utilised by Kumar et al. for semi-synthetic library production (Kumar et al., 2017), however it had initially proven problematic for amide analogue generation. Attempts to form serrulatane-based amides by Kumar et al. under standard conditions using coupling agents such as EDCI (Dunetz et al., 2016), T3P (Dunetz et al., 2016; Montalbetti and Falque, 2005; Valeur and Bradley, 2009), oxalyl chloride (Itoh et al., 2007) and HBTU (Dunetz et al., 2016; Montalbetti and Falque, 2005; Valeur and Bradley, 2009) were unsuccessful. However, a one-pot process wherein a mixed anhydride was prepared *in situ* and subsequently reacted with the desired amines finally proved successful. Unfortunately, this particular library had multiple "Rule of Five" violations, and none of the amidations afforded yields >18%; a better semi-synthetic method with higher yields and products that contained minimal or no "Rule of Five" violations was sought. Due to our group's past success with the generation of methyl esters of NPs and subsequent aminolysis with primary amines to generate amide analogues (Kumar et al., 2015), we thought the permethylation of scaffold **1** using simple and rapid methodology reported previously may be worthwhile pursuing (Ciucanu and Kerek, 1984). This high-yielding method, which uses MeI, NaOH, and DMSO at room temperature for only 6 min, had previously been shown to generate *O*-methyl ethers of various sugar moieties, and we predicted this method of permethylation should methylate all hydroxyl groups and the carboxylic acid found in serrulatane scaffold **1**. We initially ran this reaction for 1 h using standard conditions, which afforded both the tri-methyl product, 3-hydroxy-7,8-dimethoxyserrulat-14-en-19-oic acid methyl ester (**3**, 246.2 mg, 64.4% yield) and tetra-methyl product, 3,7,8-trimethoxyserrulat-14-en-19-oic acid methyl ester (**4**, 33.0 mg, 8.3% yield) in moderate yields (Scheme 1). The generation of the methyl ester analogues **3** and **4** was predicted to enable solvent-free aminolysis under mild conditions to produce the desired serrulatane amide analogues. Prior to the amidation chemistry being attempted, both methylated products were fully characterised using NMR and MS data, which confirmed the chemical structures of the products.

Prior to aminolysis with commercially available primary amines (Barnes et al., 2012; Choomuenwai et al., 2012; Egbewande et al., 2017; Kumar et al., 2015, 2017), two virtual analogue amide libraries based on **3** and **4** were constructed and analysed for their physicochemical parameters using ChemDraw Ultra 12.0.2 software and compared with Lipinski's "Rule of Five" (Mw ≤500, log P ≤5, HBD ≤5 and HBA ≤10) for drug-like properties (Fig. S97; Table S98) (Lipinski et al., 1997). Due to the lower yields of the tetra-methylated product (**4**) and a higher number of "Rule of Five" violations for the virtual library based on this scaffold, a 12-membered amide library (**5**–**16**) was synthesised by reacting the tri-methyl scaffold (**3**) with a diverse series of purchased primary amines, as shown in Fig. 1.

Before reacting the serrulatane scaffold **3** with all 11 selected amines, we decided to optimise the aminolysis reaction using *n*-propylamine based on reported methods (Karis et al., 2007), with work-up and purification of each reaction by silica SPE chromatography. Compound **3** was reacted with *n*-propylamine at room temperature (r.t) and 40 °C, both for 16 h and 48 h. It was decided that reaction conditions would not be run at higher temperatures than 40 °C, since many of the chosen commercial amines for this chemistry had low boiling points (e.g., *n*-propylamine, bp = 48 °C), which could make the chemistry problematic or fail due to loss of reagent over time or degradation of the starting material. From the optimisation reaction outcomes, it was evident that some heat was needed to improve yields (e.g., r. t, 16 h = 12% yield; 40 °C, 16 h = 26% yield), and longer run times also marginally increased yields (e.g., r. t, 48 h = 20% yield; 40 °C, 48 h = 29% yield). As the product of interest obtained during these optimisation reactions was in high-purity and sufficient yields to allow for detailed biological evaluations in the planned *in vitro* assays, further optimisation was not pursued in this study. Therefore, based on the product yields, all remaining amidations were conducted for 48 h at 40 °C (Scheme 1). While the amide library based on scaffold **4** was not prioritised, we were curious to know if the optimised amidation reaction with *n*-propylamine generated higher yields with **4** compared to scaffold **3**. From the additional and only aminolysis reaction performed on scaffold **4**, it was noted that similar yields were obtained when *n*-propylamine was reacted with each methylated scaffold **3** and **4**, the desired amide products **5** and **6**, were obtained in 29% and 30% yields, respectively.

In summary, a total of 12 amide analogues (**5**–**16**) were semi-synthesised *via* an optimised aminolysis procedure. Reactions were performed using 20 mg of methylated scaffold **3**, with excess primary amine added (500 μL); reactions products were purified using silica SPE (CH_2Cl_2/MeOH and/or *n*-hexane/EtOAc) with yields ranging from 4 to 56%, and purities >95%. Furthermore, all amide structures were fully elucidated and characterised using 1D/2D NMR, UV, $[\alpha]_D$, ECD, and MS. The structure elucidation studies on one amide are detailed below. The HRESIMS spectrum of analogue **5** revealed an ion at *m/z* 440.2771 $[M+Na]^+$ that allowed a molecular formula of $C_{25}H_{39}NO_4$ to be assigned to this compound. The ^1H NMR spectrum of compound **5** indicated the presence of one aromatic proton (δ_H 7.31), one olefinic proton (δ_H 5.17), four methine (δ_H 2.18, 2.90, 3.31, 4.22), five methylene (δ_H 2.08/1.60, 1.57/1.38, 1.66, 2.06, 3.40/3.34), seven methyl (δ_H 3.88, 3.88, 1.68, 1.62, 1.26, 1.01, 0.46). The ^{13}C NMR and the edited HSQC spectra



Scheme 1. Synthesis of poly-methylated scaffolds (**3** and **4**) and the amide library (**5**–**16**).

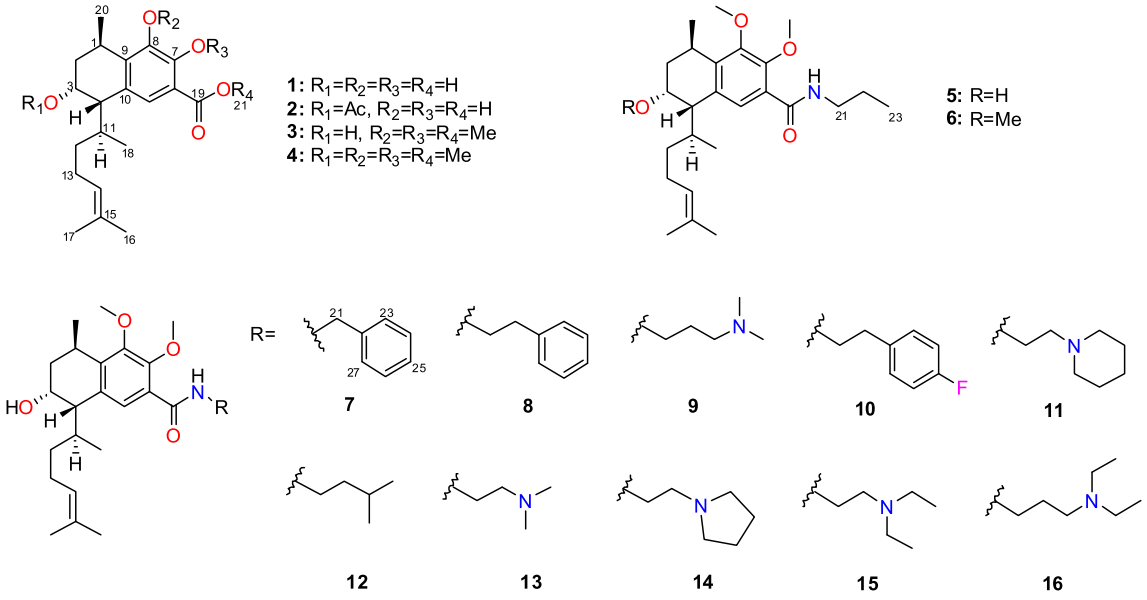


Fig. 1. Chemical structures of the targeted NP diterpenoid scaffolds (**1**–**2**), methylated products (**3**–**4**) and the amide library (**5**–**16**).

indicated a total of 25 carbons including seven methyls (δ_C 61.6, 61.0, 24.0, 25.9, 19.4, 17.8, 11.8), five methylenes (δ_C 35.5, 40.2, 27.3, 42.5, 23.8), four methines (δ_C 30.6, 31.8, 50.3, 67.4), two olefinic carbons (δ_C 125.9, 131.9), six aromatic carbons (δ_C 126.1, 127.1, 135.6, 141.2, 150.8, 152.4), one carbonyl carbon (δ_C 168.1). Both the ^{1}H and ^{13}C NMR spectra signal count along with the HRMS strongly suggested the desired product had formed (Fig. S23–S24, S85). Furthermore, two spin systems were identified for **5** following COSY data analysis, one of which included a propyl chain. Importantly, the two doublet of triplets at δ_H 3.40 (J = 13.2, 7.1 Hz, H-21a) and δ_H 3.34 (J = 13.2, 7.1 Hz, H-21b), which formed part of this *n*-propyl chain, showed strong three-bond HMBC correlations to the one carbonyl signal in this compound, which resonated at δ_C 168.1 (C-19). These data confirmed the presence and location of the amide linkage found in compound **5**. The remaining NMR signals were assigned following comparison of the chemical shifts with the previously reported NP, 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**1**), and further analysis of the 2D NMR data. Key COSY, HMBC and ROESY correlations are shown below (Fig. 2). Collectively, these data confirmed the chemical structure of compound **5**.

As part of our on-going research into the discovery of natural compounds or derivatives from nature that may hold promise as anthelmintic lead drugs (Geary, 2016), we tested all natural and

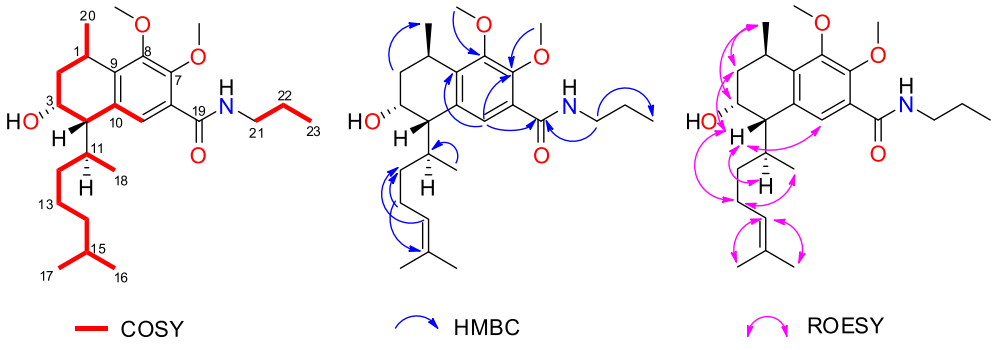


Fig. 2. Key COSY, HMBC and ROESY correlations of analogue **5**.

semi-synthetic analogues for activity against *H. contortus*, a highly pathogenic, parasitic nematode of ruminants. No *Eremophila*-derived compounds have been reported to date to have anthelmintic activity. Screening of compounds **1**–**16** on exsheathed third-stage larvae (xL3s) of *H. contortus* suggested that none of the compounds potently inhibited xL3s motility, although the NP 3-acetoxy-7,8-dihydroxyserrulat-14-e-n-19-oic acid (**2**) reduced xL3s motility by 45.1% at 100 μM after 72 h (Figure S99a). While none of the compounds inhibited larval development from xL3 to fourth-stage larvae (L4s), nine of 16 compounds (**2**–**8**, **10**, **12**) induced a *skinny* (*Skn*) phenotype in L4s after seven days of treatment at 100 μM. The *Skn* phenotype was characterised by a reduced thickness of larvae with reference to wild-type (WT) larvae exposed to DMSO alone (which had no apparent abnormality) (Taki et al., 2020). A subsequent assessment of the 16 compounds in a dose-response assay revealed that compound **3** induced a *Skn* phenotype in 78.4% of L4s at 100 μM after seven days (IC_{50} = 13.5 μM). Compound **3** reduced the mid-body width of treated L4s to 8.8 ± 1.5 μm as compared with 16.3 ± 1.5 μm for untreated WT *H. contortus*, as shown in Fig. 3.

Due to the previously reported anti-microbial activity for some *Eremophila*-derived diterpenoid serrulatanes (Barnes et al., 2013; Biva et al., 2016; Ndi et al., 2007; Smith et al., 2007), compounds **1**–**16** were tested by the Community for Open Antimicrobial Drug Discovery (CO-ADD) (Blaskovich et al., 2015; Zuegg et al., 2020) against the "ESKAPE" pathogens: *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Staphylococcus aureus* (MRSA), and the yeasts *Candida albicans* and *Cryptococcus neoformans*. None of the compounds showed growth inhibitory activity at 32 μg/mL (or ~20 μM). Furthermore, *in vitro* cytotoxicity testing revealed that none of the 16 serrulatane-based compounds were toxic to HepG2 human liver carcinoma cells, with only compound **10** exhibiting an CC_{50} (concentration causing 50% cytotoxicity) of 39.6 μM and others displaying an CC_{50} of ≥50 μM. The toxicity of these compounds was also evaluated by measuring cytotoxicity against HEK293 human embryonic kidney cells and haemolytic activity against human red blood cells. All compounds were defined as non-toxic as both the HEK293 CC_{50} and HC_{10} (concentration causing 10% haemolytic activity) were >32 μg/mL (or >20 μM).

Compounds were also evaluated for their *in vitro* effects on suppressing HIV production using the established J-Lat cell line model of HIV latency (Jordan et al., 2003; Schonhofer et al., 2021). Briefly, J-Lat cells are a T cell line which contains a latent, non-infectious latent HIV provirus fused to a green fluorescent protein (GFP) reporter. Virus expression can be induced by the treatment with known proviral, HIV latency-reversing agents such as phorbol 12-myristate 13-acetate (PMA), which is subsequently monitored by GFP expression in live cells by flow cytometry (Fig. 4). In the absence of proviral stimulation, we observed a spontaneous but low-level expression of GFP-tagged virus in 1.8 ± 0.4% of cells (Fig. 4A), which increased to 70.3 ± 5.6% following 24 h treatment with 50 nM PMA (Fig. 4B). PMA-induced virus expression, however, was inhibited by >80% after co-treatment with 100 μM of the NP, 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**2**) (Fig. 4C). Across all semi-synthetic compounds, we identified three compounds (**3, 5, 10**) which inhibited >50% of provirus expression at 100 μM (Table S101). While viral inhibition was observed by **2** and **5** only at 100 μM, dose-dependent inhibition was observed for **3** and **10** (Fig. 4D), resulting in EC_{50} values of 38.3 ± 3.2 and 51.7 ± 13.8 μM, respectively. Similar results were observed when cells were monitored for expression of the viral protein $p24^{Gag}$, supporting that compounds were also inhibiting viral protein production as opposed to simply quenching GFP fluorescence (data not shown). These results indicate that a subset of compounds can inhibit *in vitro* HIV expression following viral integration and may potentially promote HIV latency.

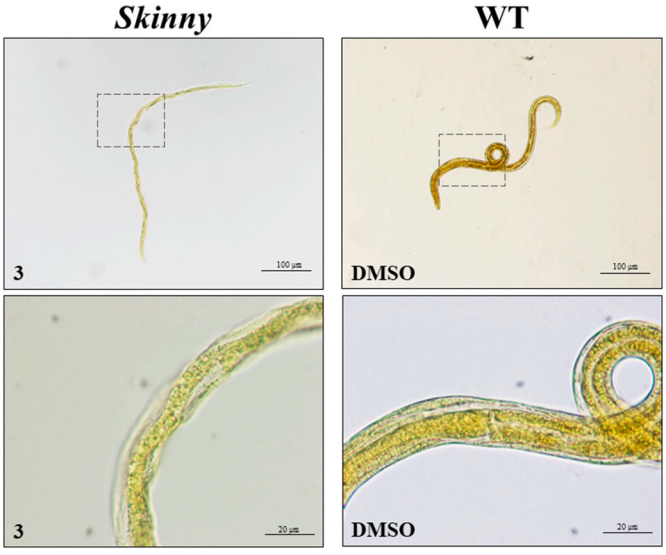


Fig. 3. Microscopic images of the *skinny* (*Skn*) phenotype in L4s induced by compound **3** compared with the wild-type (WT) phenotype control (cultured in culture medium + 0.25% DMSO); the bottom panels enlarged from the dashed/boxed areas in the upper panels.

3. Conclusions

In summary, the targeted diterpenoid scaffolds **1** and **2** were isolated from the aerial parts of *E. microtheca*, and the most abundant scaffold **1** was converted to poly-methylated derivatives **3** and **4**. The methylated product **3** was subsequently used to generate a previously undescribed 12-membered amide library (**5**–**16**). The preliminary biological screening data for all 16 compounds identified that the NP scaffold **1** showed no activity in these assays, while the tri-methyl derivative (**3**) displayed low-to-moderate activities in both anthelmintic and anti-HIV expression assays. For the amide series, the 4-fluorophenethylamide analogue (**10**) displayed weak inhibitory activity against HepG2 viability and HIV viral production. This finding was similar to the previous serrulatane-based semi-synthetic studies where a 4-fluorophenethylamide analogue was reported as the most active anti-malarial derivative (Kumar et al., 2017). Although none of the compounds were significantly active in these assays, further structure activity relationship studies are warranted. The NP 3-acetoxy-7,8-dihydroxyserrulat-14-e-n-19-oic acid (**2**), and semi-synthetic derivatives **3** and **10** could be used as leads for further medicinal chemistry optimisation in order to improve potency and selectivity against helminths, HIV, and perhaps other pathogens. These studies also further support the significance of the use of NP scaffolds for screening library generation in drug discovery and chemical biology research.

4. Experimental section

4.1. General experimental procedures

Specific rotations were determined on a JASCO P-1020 polarimeter. UV spectra were recorded using a JASCO V-650 UV/Vis spectrophotometer. ECD spectra were obtained on a JASCO J-715 spectropolarimeter and processed using the software SDAR (Weeratunga et al., 2012). NMR spectra were recorded at 25 °C on a Bruker AVANCE III HD 500 or 800 MHz NMR spectrometer equipped with a cryoprobe. The ^{1}H and ^{13}C chemical shifts were referenced to solvent peaks for CD_3OD at δ_H 3.31 and δ_C 49.0, respectively. LRESIMS data was recorded on an Ultimate 3000 RS UHPLC coupled to a Thermo Fisher Scientific MSQ Plus single quadruple ESI mass spectrometer. HRESIMS data were acquired on a Bruker maXis II ETD ESI-qTOF. GRACE Davisil (35–70 μm, 60 Å) C_{18} bonded silica was packed into an open glass column (65 × 40 mm) for C_{18} flash column chromatography. GRACE Davisil C_{18} bonded silica was used for pre-adsorption work before HPLC separations, and

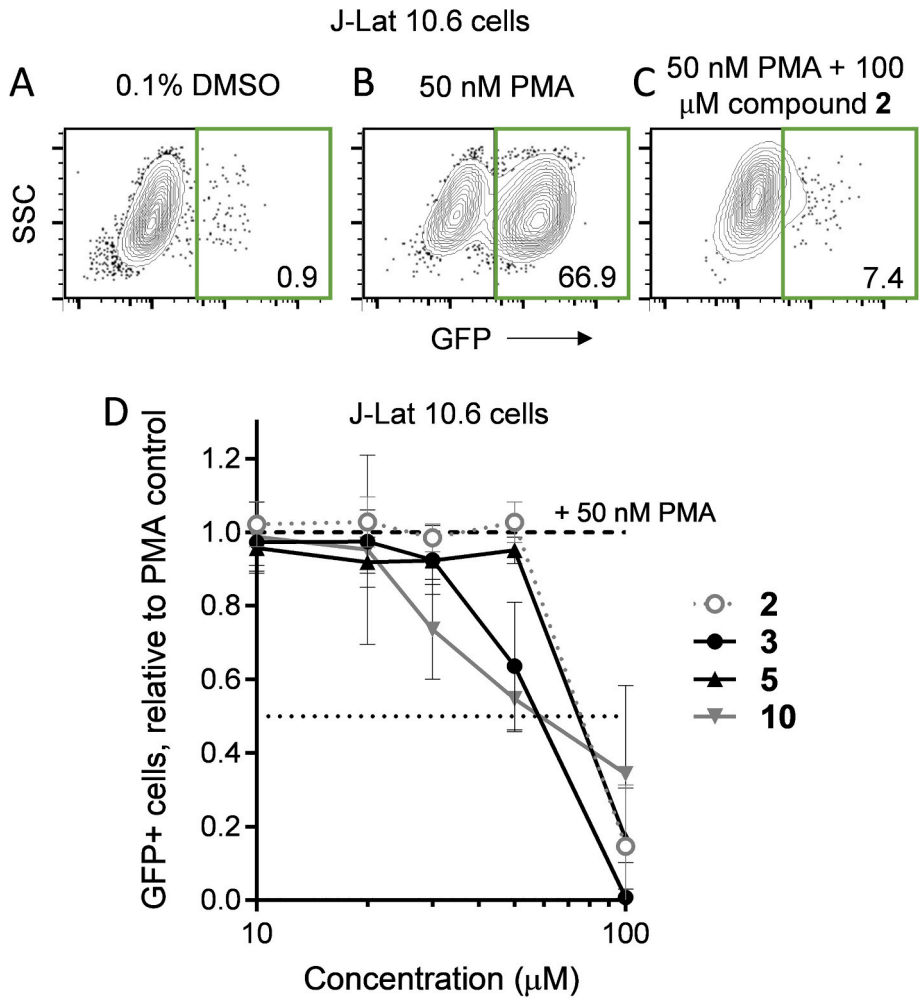


Fig. 4. Representative flow cytometry examples of GFP expression (i.e., HIV production) in J-Lat 10.6 cells in the absence of stimulation (A), treatment with 50 nM PMA (B) and PMA plus 100 μM compound **2** (C). Dose response profiles (D) of compounds on GFP expression induced by 50 nM PMA in J-Lat 10.6 cells. Data denote mean ± s.d. from three independent experiments.

the pre-adsorbed sample was packed into an Alltech stainless steel guard cartridge (10 × 30 mm). A Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC was used for semi-preparative HPLC separations. A Thermo Betasil 5 μm C_{18} column (150 × 21.2 mm) was used when performing the semi-preparative HPLC separations. The crude reaction products were purified using an ISOLUTE silica SPE cartridge (20 × 35 mm, 5 g). Merck silica gel 60 F_{254} pre-coated aluminium plates were used for thin layer chromatography (TLC) and analysed under UV light at 254 and 365 nm. The air-dried aerial parts of *Eremophila microtheca* were ground using a Fritsch Universal Cutting Mill Pulverisette 19. The plant material was extracted at room temperature using an Edwards Instrument Company Bio-line orbital shaker set to 200 rpm. All chemical reagents used throughout the experiments were purchased from Sigma-Aldrich and all solvents used for chromatography, UV, ECD, $[a]_D$ and MS were Honeywell Burdick & Jackson or Lab-Scan HPLC grade. H_2O was filtered using a Sartorius Stedium Arium® Pro VF ultrapure water system.

4.2. Plant material

Eremophila microtheca (F. Muell. ex Benth.) F. Muell. aerial parts were collected on private property at Samford, Queensland, Australia (27°22′25.3″S 152°53′11.5″E) during March 2018. Mr. Peter Bevan from the Australian Native Plant Society Australia (ANPSA) identified the plant as *E. microtheca*. After air-drying for 1 month, the plant material was ground into a fine powder ready for extraction and isolation chemistry. A voucher specimen (RAD076) has been deposited at the Griffith Institute for Drug Discovery, Griffith University, Nathan, Queensland, Australia.

4.3. Extraction and isolation

The air-dried and ground aerial parts of *E. microtheca* (1 kg) were extracted exhaustively with CH_2Cl_2 (6 × 1 L). These CH_2Cl_2 extracts were combined and dried under reduced pressure to yield a dark green amorphous powder (53 g). A portion of the CH_2Cl_2 extract (~5 g) was pre-adsorbed to C_{18} bonded silica (~5 g) then loaded onto a 10% MeOH/90% H_2O/0.1% TFA equilibrated C_{18} bonded silica flash column (65 × 40 mm). The column was subsequently flushed using a 10% stepwise elution from 10% MeOH/90% H_2O/0.1% TFA to 100% MeOH/0.1% TFA (200 mL), which resulted in 10 fractions (F1–F10). UHPLC-MS and ^{1}H NMR experiments were utilised to analyse the 10 fractions. F8 (2041.3 mg) was further purified by a C_{18} Betasil column using a linear gradient from 40% MeOH/60% H_2O/0.1% TFA to 75% MeOH/25% H_2O/0.1% TFA over 120 min at a flow rate of 9 mL/min to give 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**1**, 625.5 mg, t_R 56–65 min, 0.663% dry wt). F10 (868.9 mg) was subjected to C_{18} semi-preparative HPLC with a linear gradient from 30% MeOH/70% H_2O/0.1% TFA to 100% MeOH/0.1% TFA over 60 min at a flow rate of 9 mL/min to afford 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**2**, 52.3 mg, t_R 39

min, 0.055% dry wt).

3,7,8-Trihydroxyserrulat-14-en-19-oic acid (**1**): Stable brown gum; $[\alpha]_D^{24}$+18.7 (*c* 0.05, MeOH) (Barnes et al., 2013), $[\alpha]_D^{25}$+9.1 (*c* 0.044, MeOH); ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.09 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.23 (1H, ddd, J = 12.1, 5.2, 3.8 Hz, H-3), 3.35 (1H, ddq, J = 7.0, 1.3, 7.0 Hz, H-1), 2.83 (1H, br d, J = 5.1 Hz, H-4), 2.14 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.05 (2H, m, H-13), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.59 (1H, m, H-2b), 1.55 (1H, m, H-12a), 1.34 (1H, m, H-12b), 1.28 (3H, d, J = 7.0 Hz, H-20), 0.44 (3H, d, J = 7.0 Hz, H-18). ^{13}C NMR (CD_3OD, 200 MHz) δ_C 173.9 (C-19), 148.9 (C-7), 143.8 (C-8), 136.3 (C-9), 131.9 (C-15), 129.5 (C-10), 125.9 (C-14), 121.9 (C-5), 111.0 (C-6), 67.8 (C-3), 50.0 (C-4), 40.2 (C-12), 35.5 (C-2), 31.7 (C-11), 30.1 (C-1), 27.2 (C-13), 25.9 (C-16), 22.4 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 347 $[M-H]^-$.

3-Acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**2**): Stable brown gum, $[\alpha]_D^{24}$+3.3 (*c* 0.09, MeOH) (Barnes et al., 2013), $[\alpha]_D^{25}$+6.0 (*c* 0.033, MeOH); ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.10 (1H, s, H-5), 5.13 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 5.32 (1H, ddd, J = 11.8, 5.0, 3.7 Hz, H-3), 3.35 (1H, ddq, J = 7.0, 2.1, 7.0 Hz, H-1), 3.02 (1H, br d, J = 4.3 Hz, H-4), 2.19 (1H, ddd, J = 13.0, 12.1, 7.1 Hz, H-2a), 2.06 (1H, m, H-11), 2.04 (1H, s, H-22), 2.00 (2H, m, H-13), 1.69 (3H, s, H-16), 1.65 (1H, m, H-2b), 1.61 (3H, s, H-17), 1.45 (1H, m, H-12a), 1.30 (3H, d, J = 7.1 Hz, H-20), 1.25 (1H, m, H-12b), 0.55 (3H, d, J = 7.0 Hz, H-18). ^{13}C NMR (CD_3OD, 200 MHz) δ_C 173.8 (C-19), 149.1(C-7), 143.9 (C-8), 135.8 (C-9), 132.4 (C-15), 128.3 (C-10), 125.5 (C-14), 121.7 (C-5), 111.4 (C-6), 71.9 (C-3), 46.6 (C-4), 39.1 (C-12), 33.0 (C-2), 32.9 (C-11), 29.8 (C-1), 27.0 (C-13), 25.9 (C-16), 22.1 (C-22), 21.4 (C-20), 19.5 (C-18), 17.8 (C-17); LRESIMS *m/z* 389 $[M-H]^-$.

4.4. Methylation of 3,7,8-trihydroxyserrulat-14-en-19-oic acid (*1*)

3,7,8-Trihydroxyserrulat-14-en-19-oic acid (**1**, 341 mg, 0.9799 mmol) and NaOH (~12 equiv., 480 mg, 12 mmol) were dissolved in DMSO (5 mL), followed by the slow addition of MeI (~20 equiv., 1.2 mL, 19.3 mmol) (Ciucanu and Kerek, 1984). The reaction mixture was stirred for 1 h at room temperature and then transferred to a separatory funnel containing CH_2Cl_2 (30 mL) and H_2O (30 mL). The H_2O layer was washed with CH_2Cl_2 (2 × 30 mL) and the CH_2Cl_2-soluble material was dried under N_2. The crude product was pre-adsorbed to C_{18} bonded silica (1.5 g) and subsequently subjected to HPLC using a semi-preparative C_{18} Betasil column at a flow rate of 9 mL/min with a linear gradient of 50% MeOH/50% H_2O/0.1% TFA to 100% MeOH/0.1% TFA over 90 min. The fractions that eluted from 34 to 43 min yielded the tri-methylated compound, 3-hydroxy-7,8-dimethoxyserrulat-14-en-19-oic acid methyl ester (**3**, 246.2 mg, 64%) and the tetra-methylated compound, 3,7,8-trimethoxyserrulat-14-en-19-oic acid methyl ester (**4**, 33.0 mg, 8%), eluted from 54 to 57 min.

Compound 3: clear gum (246.2 mg, 64.4%); $[\alpha]_D^{24}$+61.6 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.61), 247 (3.91), 295 (2.82) nm; ECD λ_{ext} (MeOH) 223 ($\Delta\varepsilon$ +2.50), 252 ($\Delta\varepsilon$ +2.01), 293 ($\Delta\varepsilon$ −0.89) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.22 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.23 (1H, ddd, J = 12.1, 5.2, 3.8 Hz, H-3), 3.88 (3H, s, H-21), 3.87 (3H, s, OMe-7), 3.83 (3H, s, OMe-8), 3.31 (1H, ddq, J = 7.1, 1.6, 7.1 Hz, H-1), 2.88 (1H, br d, J = 5.2 Hz, H-4), 2.17 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.05 (2H, m, H-13), 1.69 (3H, s, H-16), 1.62 (3H, s, H-17), 1.61 (1H, m, H-2b), 1.56 (1H, m, H-12a), 1.36 (1H, m, H-12b), 1.26 (3H, d, J = 7.1 Hz, H-20), 0.47 (3H, d, J = 7.0 Hz, H-18). ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.1 (C-19), 153.1 (C-7), 152.4 (C-8), 142.3 (C-9), 135.3 (C-10), 132.0 (C-15), 127.7 (C-5), 125.8 (C-14), 124.1 (C-6), 67.3 (C-3), 61.7 (OMe-8), 61.1 (OMe-7), 52.6 (C-21), 50.2 (C-4), 40.0 (C-12), 35.6 (C-2), 31.9 (C-11), 30.5 (C-1), 27.2 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 391 $[M+H]^+$, 413 $[M+Na]^+$; HRESIMS *m/z* 391.2480 $[M+H]^+$ (calcd for $C_{23}H_{35}O_5$, 391.2479), 413.2299 $[M+Na]^+$ (calcd for $C_{23}H_{34}NaO_5$, 413.2298).

Compound 4: clear gum (33.0 mg, 8.3%); $[\alpha]_D^{24}$+43.1 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.06), 246 (3.38), 293 (2.22) nm; ECD λ_{ext} (MeOH) 214 ($\Delta\varepsilon$ +0.91), 252 ($\Delta\varepsilon$ +0.40), 292 ($\Delta\varepsilon$ −0.17) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.24 (1H, s, H-5), 5.16 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 3.88 (3H, s, H-21), 3.87 (3H, s, OMe-8), 3.83 (3H, s, OMe-7), 3.77 (1H, ddd, J = 12.0, 5.1, 3.6 Hz, H-3), 3.40 (3H, s, OMe-3), 3.31 (1H, ddq, J = 7.0, 2.0, 7.0 Hz, H-1), 3.07 (1H, br d, J = 5.1 Hz, H-4), 2.10 (1H, m, H-11), 2.06 (1H, m, H-2a), 2.04 (2H, m, H-13), 1.70 (3H, s, H-16), 1.67 (1H, dq, J = 13.1, 1.7, 1.7 Hz, H-2b), 1.63 (3H, s, H-17), 1.55 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.1 Hz, H-20), 0.47 (3H, d, J = 7.0 Hz, H-18). ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.1 (C-19), 153.1 (C-8), 152.5 (C-7), 142.5 (C-9), 134.8 (C-10), 132.3 (C-15), 127.7 (C-5), 125.7 (C-14), 124.2 (C-6), 77.4 (C-3), 61.7 (OMe-7), 61.1 (OMe-8), 52.6 (C-21), 47.2 (C-4), 39.8 (C-12), 32.9 (C-2), 31.9 (C-11), 30.3 (C-1), 27.2 (C-13), 25.9 (C-16), 23.9 (C-20), 19.2 (C-18), 17.8 (C-17); LRESIMS *m/z* 405 $[M+H]^+$, 831 $[2M+Na]^+$; HRESIMS *m/z* 405.2636 $[M+H]^+$ (calcd for $C_{24}H_{37}O_5$, 405.2636), 427.2456 $[M+Na]^+$ (calcd for $C_{24}H_{36}NaO_5$, 427.2455).

4.5. Aminolysis of 3-hydroxy-7,8-dimethoxyserrulat-14-en-19-oic acid methyl ester (*3*)

To the tri-methylated scaffold (**3**, 20 mg, 0.051 mmol) was added the desired amine (500 μL) and the mixture stirred for 48 h at 40 °C. The crude reaction was dried under N_2 and high vacuum before being pre-adsorbed to silica (~0.2 g). The crude product was purified by silica SPE using a 2.5% or 5% stepwise gradient from 100% *n*-hexane to 70% *n*-hexane/EtOAc (30 mL elution per flush) or from 100% CH_2Cl_2 to 70% CH_2Cl_2/MeOH (30 mL elution per flush). Fractions containing UV-active material were analysed by ^{1}H NMR and UHPLC-MS to determine purity. All compounds (>95%) were characterised by 1D (^{1}H and ^{13}C) and 2D NMR (COSY, HSQC, HMBC, and ROESY) and HRESIMS data analysis.

Compound 5: clear gum (5.9 mg, 29.0%); $[\alpha]_D^{24}$+20.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.74), 246 (4.11), 287 (2.64) nm; ECD λ_{ext} (MeOH) 218 ($\Delta\varepsilon$ +4.30), 251 ($\Delta\varepsilon$ −1.00), 290 ($\Delta\varepsilon$ −0.51) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.31 (1H, s, H-5), 5.17 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.1, 5.2, 3.8 Hz, H-3), 3.88 (3H, s, OMe-7), 3.88 (3H, s, OMe-8), 3.40 (1H, ddt, J = 13.2, 7.1 Hz, H-21a), 3.34 (1H, ddt, J = 13.2, 7.1 Hz, H-21b), 3.31 (1H, ddq, J = 7.0, 1.5, 7.0 Hz, H-1), 2.90 (1H, br d, J = 5.2 Hz, H-4), 2.18 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.68 (3H, s, H-16), 1.65 (2H, m, H-22), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.37 (1H, m, H-12b), 1.26 (3H, d, J = 7.0 Hz, H-20), 1.01 (3H, t, J = 7.4 Hz, H-23), 0.46 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.1 (C-19), 152.4 (C-8), 150.8 (C-7), 141.2 (C-9), 135.6 (C-10), 131.9 (C-15), 127.1 (C-5), 125.9 (C-14), 126.1 (C-6), 67.4 (C-3), 61.6 (OMe-8), 61.0 (OMe-7), 50.3 (C-4), 42.5 (C-21), 40.2 (C-12), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 23.8 (C-22), 19.4 (C-18), 17.8 (C-17), 11.8 (C-23); LRESIMS *m/z* 418 $[M+H]^+$, 857 $[2M+Na]^+$; HRESIMS *m/z* 418.2953 $[M+H]^+$ (calcd for $C_{25}H_{40}NO_4$, 418.2952), 440.2773 $[M+Na]^+$ (calcd for $C_{25}H_{39}NNaO_4$, 440.2771).

Compound 6: clear gum (4.2 mg, 29.8%); $[\alpha]_D^{24}$+23.6 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.88), 246 (4.20), 289 (2.79) nm; ECD λ_{ext} (MeOH) 224 ($\Delta\varepsilon$ +7.46), 251 ($\Delta\varepsilon$ −3.00), 291 ($\Delta\varepsilon$ −1.57) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.34 (1H, s, H-5), 5.16 (1H, tqq, J = 7.1, 1.4 Hz, H-14), 3.88 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.77 (H, ddd, J = 12.0, 5.2, 3.6 Hz, H-3), 3.41 (3H, s, OMe-3), 3.40 (1H, dd, J = 13.5, 7.2, 1.2 Hz, H-21a), 3.34 (1H, ddd, J = 13.5, 7.2, 1.2 Hz, H-21 b), 3.31 (1H, ddq, J = 7.1, 1.8, 7.1 Hz, H-1), 3.09 (1H, br d, J = 5.1 Hz, H-4), 2.10 (1H, m, H-11), 2.06 (1H, m, H-2a), 2.04 (2H, m, H-13), 1.69 (3H, s, H-16), 1.68 (1H, m, H-2b), 1.67 (1H, m, H-22a), 1.65 (1H, m, H-22b), 1.62 (3H, s, H-17), 1.56 (1H, m, H-12a), 1.40 (1H, m, H-12b), 1.27 (3H, d, J = 7.1 Hz, H-20), 1.01 (3H, t, J = 7.4 Hz, H-23), 0.46 (3H, d, J = 6.9 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.1 (C-19), 150.8 (C-7), 152.4 (C-8), 141.4 (C-9), 135.1 (C-10), 132.2 (C-15), 127.1 (C-5), 125.7 (C-14), 126.2 (C-6), 77.5 (C-3), 61.6 (OMe-7), 61.1 (OMe-8), 56.4 (OMe-3),

47.2 (C-4), 42.5(C-21), 40.0 (C-12), 33.0 (C-2), 31.8 (C-11), 30.3 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 23.8 (C-22), 19.2 (C-18), 17.8 (C-17), 11.8 (C-23); LRESIMS *m/z* 432 $[M+H]^+$, 885 $[2\,M+Na]^+$; HRESIMS *m/z* 432.3108 $[M+H]^+$ (calcd for $C_{26}H_{42}NO_4$, 432.3108), 454.2928 $[M+Na]^+$ (calcd for $C_{26}H_{41}NNaO_4$, 454.2928).

Compound 7: clear gum (1.0 mg, 4.2%); $[\alpha]_D^{24}$+35.0 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.32), 247 (3.66), 285 (0.63) nm; ECD λ_{ext} (MeOH) 222 ($\Delta\varepsilon$ +4.57), 251 ($\Delta\varepsilon$ −1.36), 290 ($\Delta\varepsilon$ −0.78) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.39 (2H, br d, J = 7.8 Hz, H-23, H-27), 7.35 (2H, m, H-24, H-26), 7.34 (1H, s, H-5), 7.27 (1H, tt, J = 7.4, 1.3 Hz, H-25), 5.17 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.65 (H, d, J = 14.8 Hz, H-21a), 4.55 (H, d, J = 14.8 Hz, H-21b), 4.23 (1H, ddd, J = 12.1, 5.3, 3.7 Hz, H-3), 3.87 (3H, s, OMe-8), 3.81 (3H, s, OMe-7), 3.31 (1H, m, H-1), 2.91 (1H, br d, J = 5.2 Hz, H-4), 2.18 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.58 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.0 Hz, H-20), 0.47 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.1 (C-19), 152.5 (C-8), 150.9 (C-7), 141.4 (C-9),140.1 (C-22), 135.7 (C-10), 131.9 (C-15),129.6 (C-24, C-26), 128.6 (C-23, C-27), 128.3 (C-25), 127.1 (C-5), 126.0 (C-6), 125.9 (C-14), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 50.3 (C-4), 44.5 (C-21), 40.1 (C-12), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 466 $[M+H]^+$, 953 $[2\,M+Na]^+$; HRESIMS *m/z* 466.2951 $[M+H]^+$ (calcd for $C_{29}H_{40}NO_4$, 466.2952), 488.2771 $[M+Na]^+$ (calcd for $C_{29}H_{39}NNaO_4$, 488.2771).

Compound 8: clear gum (2.4 mg, 9.2%); $[\alpha]_D^{24}$+22.9 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.16), 247 (3.47), 493 (2.30) nm; λ_{ext} (MeOH) 214 ($\Delta\varepsilon$ +2.83), 251 ($\Delta\varepsilon$ −0.41), 291 ($\Delta\varepsilon$ −0.17) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.32 (1H, s, H-5), 7.31 (2H, m, H-25, H-27), 7.30 (2H, m, H-24, H-28), 7.22 (1H, tt, J = 6.7, 2.1 Hz, H-26), 5.18 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.21 (1H, ddd, J = 12.1, 5.1, 3.7 Hz, H-3), 3.83 (3H, s, OMe-8), 3.72 (1H, ddd, J = 13.4, 7.2, 7.0 Hz, H-21a), 3.66 (1H, ddd, J = 13.4, 7.2, 7.0 Hz, H-21b), 3.63 (3H, s, OMe-7), 3.29 (1H, ddq, J = 7.0, 1.4, 7.0 Hz, H-1), 2.98 (1H, ddd, J = 13.7, 7.2, 7.2 Hz, H-22a), 2.93 (1H, ddd, J = 13.7, 7.2, 7.2 Hz, H-22 b), 2.89 (1H, br d, J = 5.2 Hz, H-4), 2.17 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.69 (3H, s, H-16), 1.63 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.25 (3H, d, J = 7.1 Hz, H-20), 0.44 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.0 (C-19), 150.9 (C-7), 152.4 (C-8), 141.4 (C-9), 140.5 (C-23), 135.6 (C-10), 131.9 (C-15), 129.9 (C-25, C-27), 129.6 (C-24, C-28), 127.5 (C-26), 127.2 (C-5), 125.9 (C-14), 125.5 (C-6), 67.4 (C-3), 61.3 (OMe-7), 61.0 (OMe-8), 42.0 (C-21), 50.4 (C-4), 40.2 (C-12), 36.2 (C-22), 35.4 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 480 $[M+H]^+$, 981 $[2M+Na]^+$; HRESIMS *m/z* 480.3108 $[M+H]^+$ (calcd for $C_{30}H_{42}NO_4$, 480.3108), 502.2928 $[M+Na]^+$ (calcd for $C_{30}H_{41}NNaO_4$, 502.2928).

Compound 9: clear gum (11.1 mg, 36.3%); $[\alpha]_D^{24}$+17.9 (*c* 0.29, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.70), 247 (4.06), 290 (2.39) nm; ECD λ_{ext} (MeOH) 210 ($\Delta\varepsilon$ +3.24), 249 ($\Delta\varepsilon$ −0.74), 289 ($\Delta\varepsilon$ −0.49) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.30 (1H, s, H-5), 5.17 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.2, 5.2, 3.7 Hz, H-3), 3.89 (3H, s, OMe-7), 3.88 (3H, s, OMe-8), 3.46 (1H, ddd, J = 13.5, 6.9, 6.9 Hz, H-21a), 3.44 (1H, ddd, J = 13.5, 6.9, 6.9 Hz, H-21b), 3.31 (1H, ddq, J = 7.0, 1.5, 7.0 Hz, H-1), 2.90 (1H, br d, J = 5.1 Hz, H-4), 2.48 (2H, br t, J = 7.7 Hz, H-23), 2.31 (6H, s, H-24, H-25), 2.18 (1H, m, H-11), 2.07 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.84 (2H, dtt, J = 6.9, 6.9, 7.7 Hz, H-22), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.37 (1H, m, H-12 b), 1.26 (3H, d, J = 7.1 Hz, H-20), 0.46 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.2 (C-19), 152.4 (C-8), 150.8 (C-7), 141.3 (C-9), 135.6 (C-10), 132.0 (C-15), 127.0 (C-5), 126.1 (C-6), 125.9 (C-14), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 58.2 (C-23), 50.3 (C-4), 45.4 (C-24, C-25), 40.2 (C-12), 38.9 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 28.1 (C-22), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 461 $[M+H]^+$; HRESIMS *m/z* 461.3374 $[M+H]^+$ (calcd for $C_{27}H_{45}N_2O_4$, 461.3374), 483.3190 $[M+Na]^+$ (calcd for $C_{27}H_{44}N_2NaO_4$, 483.3193).

Compound 10: clear gum (6.5 mg, 19.7%); $[\alpha]_D^{24}$+17.9 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 211 (4.78), 248 (4.14), 289 (1.70) nm; ECD λ_{ext} (MeOH) 210 ($\Delta\varepsilon$ +1.04), 251 ($\Delta\varepsilon$ −0.23), 294 ($\Delta\varepsilon$ −0.14) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.31 (1H, s, H-5), 7.03 (2H, m, H-25, H-27), 7.30 (2H, m, H-24, H-28), 5.18 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.21 (1H, ddd, J = 12.2, 5.1, 3.6 Hz, H-3), 3.83 (3H, s, OMe-8), 3.69 (3H, s, OMe-7), 3.68 (1H, ddd, J = 13.4, 7.2, 7.2 Hz, H-21a), 3.63 (1H, ddd, J = 13.4, 7.2, 7.2 Hz, H-21b), 3.29 (1H, ddq, J = 7.1, 1.5, 7.1 Hz, H-1), 2.95 (1H, m, H-22a), 2.92 (1H, m, H-22b), 2.89 (1H, br d, J = 5.2 Hz, H-4), 2.17 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.69 (3H, s, H-16), 1.62 (3H, s, H-17), 1.59 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.37 (1H, m, H-12b), 1.25 (3H, d, J = 7.1 Hz, H-20), 0.44 (3H, d, J = 7.1 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.0 (C-19), 163.1 ($^1J_{CF}$ = 242.9 Hz, C-26), 150.9 (C-7), 152.4 (C-8), 141.4 (C-9), 136.5 (C-23), 135.6 (C-10), 131.9 (C-15), 131.6 ($^3J_{CF}$ = 8.1 Hz, C-24, C-28), 127.1 (C-5), 125.9 (C-14), 125.7 (C-6), 116.2 ($^2J_{CF}$ = 21.2 Hz, C-25, C-27), 67.4 (C-3), 61.4 (OMe-7), 61.0 (OMe-8), 50.4 (C-4), 42.0 (C-21), 40.2 (C-12), 35.6 (C-22), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 498 $[M+H]^+$; HRESIMS *m/z* 498.3013 $[M+H]^+$ (calcd for $C_{30}H_{41}FNO_4$, 498.3014), 520.2833 $[M+Na]^+$ (calcd for $C_{30}H_{40}FNNaO_4$, 520.2834).

Compound 11: clear gum (4.8 mg, 17.7%); $[\alpha]_D^{24}$+24.0 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.81), 248 (4.20), 290 (2.78) nm; ECD λ_{ext} (MeOH) 210 ($\Delta\varepsilon$ +14.78), 251 ($\Delta\varepsilon$ −3.14), 292 ($\Delta\varepsilon$ −1.55) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.38 (1H, s, H-5), 5.17 (1H, tqq, J = 7.0, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.3, 5.0, 3.7 Hz, H-3), 3.91 (3H, s, OMe-7), 3.88 (3H, s, OMe-8), 3.57 (1H, ddd, J = 13.7, 6.7, 6.7 Hz, H-21a), 3.53 (1H, ddd, J = 13.7, 6.7, 6.7 Hz, H-21b), 3.31 (1H, ddq, J = 7.0, 1.4, 7.0 Hz, H-1), 2.91 (1H, br d, J = 5.2 Hz, H-4), 2.58 (2H, t, J = 6.6 Hz, H-22), 2.51 (4H, m, H-23, H-27), 2.18 (1H, m, H-11), 2.07 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.68 (3H, s, H-16), 1.64 (4H, tt, J = 5.8, 5.8 Hz, H-24, H-26), 1.62 (3H, s, H-17), 1.61 (1H, m, H-2b), 1.59 (1H, m, H-12a), 1.50 (2H, br m, H-25), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.1 Hz, H-20), 0.45 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 167.8 (C-19), 152.5 (C-8), 151.1 (C-7), 141.5 (C-9), 135.6 (C-10), 131.9 (C-15), 127.3 (C-5), 125.9 (C-14), 125.5 (C-6), 67.4 (C-3), 61.7 (OMe-7), 61.0 (OMe-8), 58.6 (C-22), 55.5 (C-23, C-27), 50.4 (C-4), 40.2 (C-12), 37.7 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 26.9 (C-24, C-26), 25.9 (C-16), 25.3 (C-25), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 487 $[M+H]^+$; HRESIMS *m/z* 487.3530 $[M+H]^+$ (calcd for $C_{29}H_{47}N_2O_4$, 487.3530), 509.3345 $[M+Na]^+$ (calcd for $C_{29}H_{46}N_2NaO_4$, 509.3350).

Compound 12: clear gum (10.5 mg, 36.5%); $[\alpha]_D^{24}$+21.7 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.87), 247 (4.20), 287 (2.53) nm; ECD λ_{ext} (MeOH) 210 ($\Delta\varepsilon$ +4.84), 251 ($\Delta\varepsilon$ −0.78), 292 ($\Delta\varepsilon$ −0.36) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.30 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.1, 5.1, 3.7 Hz, H-3), 3.87 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.47 (1H, ddd, J = 13.4, 7.3, 7.3 Hz, H-21a), 3.40 (1H, ddd, J = 13.4, 7.3, 7.3 Hz, H-21b), 3.31 (1H, dqq, J = 7.1, 1.5, 7.1 Hz, H-1), 2.90 (1H, br d, J = 5.1 Hz, H-4), 2.18 (1H, m, H-11), 2.07 (1H, m, H-2a), 2.05 (2H, m, H-13), 1.71 (1H, tqq, J = 7.3, 6.7, 6.7 Hz, H-23), 1.69 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.53 (2H, ddd, J = 7.3, 7.3, 7.3 Hz, H-22), 1.38 (1H, m, H-12b), 1.26 (3H, d, J = 7.1 Hz, H-20), 0.99 (3H, dd, J = 6.7, 1.2 Hz, H-24), 0.98 (3H, dd, J = 6.7, 1.2 Hz, H-25), 0.46 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.0 (C-19), 152.4 (C-8), 150.8 (C-7), 141.2 (C-9), 135.6 (C-10), 131.9 (C-15), 127.0 (C-5), 126.1 (C-6), 125.9 (C-14), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 50.3 (C-4), 40.2 (C-12), 39.5 (C-22), 39.0 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 27.1 (C-23), 25.9 (C-16), 24.0 (C-20), 22.9 (C-24, C-25), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 446 $[M+H]^+$, 913 $[2M+Na]^+$; HRESIMS *m/z* 446.3264 $[M+H]^+$ (calcd for $C_{27}H_{44}NO_4$, 446.3265), 468.3083 $[M+Na]^+$ (calcd for $C_{27}H_{43}NNaO_4$, 468.3084).

Compound 13: clear gum (3.9 mg, 15.5%); $[\alpha]_D^{24}$+25.2 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 213 (5.08), 247 (4.41), 290 (3.37) nm; ECD λ_{ext} (MeOH) 212 ($\Delta\varepsilon$ +24.35), 251 ($\Delta\varepsilon$ −5.12), 292 ($\Delta\varepsilon$ −2.44) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.38 (1H, s, H-5), 5.17 (1H, tqq, J = 7.2, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.2, 5.1, 3.6 Hz, H-3), 3.89 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.55 (1H, dt, J = 13.6, 6.7 Hz, H-21a), 3.53 (1H, dt, J = 13.6, 6.7 Hz, H-21b), 3.31 (1H, dqq, J = 7.1, 1.4, 7.1 Hz, H-1), 2.91 (1H, br d, J = 5.2 Hz, H-4), 2.57 (2H, t, J = 6.7 Hz, H-22), 2.32 (6H, s, H-23, H-24), 2.18 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.0 Hz, H-20), 0.45 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 167.9 (C-19), 152.5 (C-8), 151.1 (C-7), 141.5 (C-9), 135.6 (C-10), 131.9 (C-15), 127.3 (C-5), 125.4 (C-6), 125.9 (C-14), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 59.0 (C-22), 50.4 (C-4), 45.5 (C-23, C-24), 40.2 (C-12), 38.4 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 447 $[M+H]^+$; HRESIMS *m/z* 447.3217 $[M+H]^+$ (calcd for $C_{26}H_{43}N_2O_4$, 447.3217), 469.3032 $[M+Na]^+$ (calcd for $C_{26}H_{42}N_2NaO_4$, 469.3037).

Compound 14: clear gum (6.0 mg, 21.6%); $[\alpha]_D^{24}$+26.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.17), 248 (3.54), 292 (1.94) nm; ECD λ_{ext} (MeOH) 212 ($\Delta\varepsilon$ +12.13), 251 ($\Delta\varepsilon$ −2.44), 291 ($\Delta\varepsilon$ −1.24) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.37 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.1, 5.1, 3.6 Hz, H-3), 3.89 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.58 (1H, m, H-21a), 3.56 (1H, m, H-21b), 3.31 (1H, ddq, J = 7.1, 1.4, 7.1 Hz, H-1), 2.91 (1H, br d, J = 5.1 Hz, H-4), 2.75 (1H, m, H-22a), 2.73 (1H, m, H-22b), 2.64 (4H, br m, H-23, H-26), 2.18 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.06 (2H, m, H-13), 1.84 (4H, m, H-24, H-25), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.58 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.1 Hz, H-20), 0.45 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 167.9 (C-19), 152.5 (C-8), 151.0 (C-7), 141.5 (C-9), 135.6 (C-10), 131.9 (C-15), 127.3 (C-5), 125.9 (C-14), 125.5 (C-6), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 55.9 (C-22), 55.0 (C-23, C-26), 50.4 (C-4), 40.2 (C-12), 39.6 (C-21), 35.5 (C-2), 31.8 (C- 11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.3 (C-24, C-25), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17); LRESIMS *m/z* 473 $[M+H]^+$; HRESIMS *m/z* 473.3372 $[M+H]^+$ (calcd for $C_{28}H_{45}N_2O_4$, 473.3374).

Compound 15: clear gum (15.1 mg, 55.7%); $[\alpha]_D^{24}$+21.4 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.40), 248 (3.77), 289 (1.94) nm; ECD λ_{ext} (MeOH) 210 ($\Delta\varepsilon$ +3.16), 254 ($\Delta\varepsilon$ −0.64), 292 ($\Delta\varepsilon$ −0.57) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.38 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.23 (1H, ddd, J = 12.2, 5.3, 3.6 Hz, H-3), 3.90 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.51 (2H, t, J = 6.7 Hz, H-21), 3.31 (1H, ddq, J = 7.0, 1.5, 7.1 Hz, H-1), 2.91 (1H, br d, J = 5.2 Hz, H-4), 2.71 (2H, t, J = 6.7 Hz, H-22), 2.64 (4H, m, H-23, H-25), 2.18 (1H, m, H-11), 2.08 (1H, m, H-2a), 2.05 (2H, m, H-13), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.61 (1H, m, H-2b), 1.58 (1H, m, H-12a), 1.38 (1H, m, H-12b), 1.27 (3H, d, J = 7.1 Hz, H-20), 0.45 (3H, d, J = 7.0 Hz, H-18), 1.09 (6H, t, J = 7.2 Hz, H-24, H-26); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 167.9 (C-19), 152.5 (C-8), 151.0 (C-7), 141.5 (C-9), 135.6 (C-10), 131.9 (C-15), 127.3 (C-5), 125.9 (C-14), 125.5 (C-6), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 52.5 (C-22), 50.4 (C-4), 48.0 (C-23, C-25), 40.2 (C-12), 38.4 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17), 11.9 (C-24, C-26); LRESIMS *m/z* 475 $[M+H]^+$; HRESIMS *m/z* 475.3531 $[M+H]^+$ (calcd for $C_{28}H_{47}N_2O_4$, 475.3530).

Compound 16: clear gum (4.5 mg, 18.3%); $[\alpha]_D^{24}$+28.3 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.87), 248 (4.25), 290 (3.05) nm; ECD λ_{ext} (MeOH) 211 ($\Delta\varepsilon$ +12.85), 252 ($\Delta\varepsilon$ −1.86), 291 ($\Delta\varepsilon$ −0.92) nm; ^{1}H NMR (CD_3OD, 800 MHz) δ_H 7.28 (1H, s, H-5), 5.17 (1H, tqq, J = 7.1, 1.4, 1.4 Hz, H-14), 4.22 (1H, ddd, J = 12.0, 5.1, 3.6 Hz, H-3), 3.88 (3H, s, OMe-7), 3.87 (3H, s, OMe-8), 3.44 (2H, t, J = 6.8 Hz, H-21), 3.31 (1H, ddq, J = 7.0, 1.5, 7.0 Hz, H-1), 2.90 (1H, br d, J = 5.2 Hz, H-4), 2.60 (2H, t, J = 7.7 Hz, H-23), 2.59 (4H, q, J = 7.2 Hz, H-24, H-26), 2.18 (1H, m, H-11), 2.07 (1H, m, H-2a), 2.05 (2H, m, H-13), 1.81 (2H, m, H-22), 1.68 (3H, s, H-16), 1.62 (3H, s, H-17), 1.60 (1H, m, H-2b), 1.57 (1H, m, H-12a), 1.37 (1H, m, H-12b), 1.26 (3H, d, J = 7.0 Hz, H-20), 1.06 (6H, t, J = 7.2 Hz, H-25, H-27), 0.45 (3H, d, J = 7.0 Hz, H-18); ^{13}C NMR (CD_3OD, 200 MHz) δ_C 168.3 (C-19), 152.4 (C-8), 150.7 (C-7), 141.2 (C-9), 135.6 (C-10), 131.9 (C-15), 127.0 (C-5), 126.4 (C-6), 125.9 (C-14), 67.4 (C-3), 61.6 (OMe-7), 61.0 (OMe-8), 51.4 (C-23), 50.4 (C-4), 47.8 (C-24, C-26), 40.2 (C-12), 39.3 (C-21), 35.5 (C-2), 31.8 (C-11), 30.5 (C-1), 27.3 (C-13), 27.2 (C-22), 25.9 (C-16), 24.0 (C-20), 19.4 (C-18), 17.8 (C-17), 11.3 (C-25, C-27); LRESIMS *m/z* 489 $[M+H]^+$; HRESIMS *m/z* 489.3687 $[M+H]^+$ (calcd for $C_{29}H_{49}N_2O_4$, 489.3687).

4.6. Anthelmintic assays

Compounds were screened using established assays for *Haemonchus contortus* in a dose-dependent manner at (decreasing) concentrations from 100 μM to 0.76 nM (Frei et al., 2020; Śliwka et al., 2016; Weise, 1977). Two positive control-compounds, monepantel and moxidectin, were prepared in the same manner as test compounds, and culture medium with 0.25% DMSO as a negative control. In brief, the xL3s were added to individual wells containing serially two-fold diluted compounds at a density of 300 per well. All plates were incubated at 38 °C and 10% (v/v) CO_2 with >90% relative humidity. The motility was measured and analysed after 72 h of incubation. Motility reduction in xL3s was verified by determining the half maximum inhibitory concentrations (IC_{50}) calculated from dose-response curves at 72 h. Plates containing xL3s were incubated for four more days, and individual compounds were also tested for their inhibition of development from the xL3 stage to the L4 stage *in vitro*. The rate of L4s development was examined microscopically; L4s have a well-developed mouth and pharynx (Weise, 1977). All compounds were tested in triplicate in both the xL3s motility and L4s development assays, and results were repeated three times on separate days. Data (Mi) from each assay were converted to a percentage compared with the negative (DMSO) control. To determine the IC_{50} values, compound concentrations were $\log_{10}$-transformed, fitted using a variable slope four-parameter equation, constraining the top value to 100% using a least squares (ordinary) fit model employing the GraphPad Prism (v. 8.4.3) software.

4.7. Anti-microbial assays

All screening assays were carried out following previously described procedures (Frei et al., 2020). Compounds were prepared in DMSO and water, and serially diluted 1:2 fold to testing concentrations ranging from 32 to 0.25 μg/mL (or 20 to 0.15 μM), with a final DMSO concentration to a maximum of 0.5% in all assays.

The anti-bacterial assays were performed in Cation-adjusted Mueller Hinton broth (CaMHB) and Yeast Nitrogen Base media was used for anti-fungal assays. The bacterial and yeast suspension were added to each well of the compound-containing plates, giving a final cell density of 5×10^5 CFU/mL and 2.5×10^3 CFU/mL, respectively. Final assay volumes were 50 μL and the growth inhibition was determined following 18 h incubation at 37 °C for bacteria and 36 h incubation at 35 °C for yeast, without shaking. The inhibition was visualised by optical density reading at 600 nm (OD_{600}) for bacteria and 630 nm (OD_{630}) for *C. albicans*. The growth inhibition of *C. neoformans* was determined by measuring the difference in absorbance between 600 and 570 nm ($OD_{600-570}$), after the addition of resazurin and further incubation at 35 °C for 2 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration that inhibited ≥80% of the bacteria and fungi growth. Colistin sulfate and vancomycin HCl were used as positive bacterial inhibitor standards for Gram-negative and Gram-positive bacteria, respectively. Fluconazole was used as positive fungal inhibitor standard for *C. albicans* and *C. neoformans*.

4.8. Cytotoxicity and haemolysis assays

The cytotoxicity of the compounds was evaluated using previously described methods (Frei et al., 2020). HEK293 human embryonic kidney cells were added to compound-containing plates to give a final density of 5000 cells/well in a final volume of 50 μL, using DMEM supplemented with 10% FBS. The plates were incubated for 20 h at 37 °C in 5% CO_2. The cell viability was assessed by resazurin assay and the fluorescence signal at excitation/emission: 560/590 nm was measured. Tamoxifen was used as positive cytotoxicity standard and CC_{50} (concentration at 50% cytotoxicity) was calculated by curve-fitting the inhibition values *vs*. log (concentration) using sigmoidal dose-response function, with variable fitting values for bottom, top and slope.

The haemolytic activity was tested following previously described procedures (Frei et al., 2020). Briefly, the human whole blood was washed three times with three volumes of 0.9% NaCl and resuspended in a concentration of 0.5×10^8 cells/mL. The washed cells were then added to the compound-containing plates for a final volume of 50 μL, shaken for 10 min and incubated for 1 h at 37 °C. The plates were centrifuged at 1000 g for 10 min to pellet cells and debris after incubation, 25 μL of the supernatant was then transferred to a polystyrene 384-well assay plate. Haemolysis was determined by measuring the supernatant absorbance at 405 mm. Melittin served as positive haemolytic standard and HC_{10} (concentration at 10% haemolytic activity) was calculated by curve-fitting the inhibition values *vs*. log (concentration) using sigmoidal dose-response function, with variable fitting values for bottom, top and slope.

4.9. Cytotoxicity of compounds against HepG2 cells

HepG2 human liver carcinoma cells were seeded into 96-well plates at a density of 1×10^5 cells per well in 80 μL of Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B (DMEM*), and incubated overnight at 37 °C and 5% (v/v) CO_2 with >90% relative humidity. After 16 h, the cells were treated with serially two-fold diluted compounds (20 μL), at final concentrations ranging from 100 μM to 1.56 μM. Doxorubicin (10 μM) served as a positive control and DMEM* + 0.25% DMSO (vehicle) used as a negative control, were applied to wells of a 96-well microtitre plates. Monepantel was serially diluted and prepared in the same manner as test compounds as a non-toxic compound reference. After 48 h of treatment, the cellular viability was measured by using the crystal violet staining assay (Śliwka et al., 2016). The absorbance at 595 nm of treated cells was compared with the absorbance of the controls, which cells were exposed only to the vehicle and were considered as 100% viable. The experiment was performed once in triplicates. To determine the CC_{50} values, compounds concentrations were log_{10}-transformed, fitted using a variable slope four-parameter equation using a least squares (ordinary) fit model using the GraphPad Prism (v. 8.4.3) software.

4.10. HIV-1 latency reversal assay

J-Lat 10.6 cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH [contributed by Dr. Eric Verdin (Jordan et al., 2003)] and cultured in R10+ media (RPMI 1640 with HEPES and L-Glutamine, 10% Fetal Bovine Serum, 100 U of penicillin/mL, and 100 μg/mL of streptomycin). Cells were seeded in 96-well plates at 2×10^5 cells/mL, co-treated with PMA (≥99% HPMC) and/or compounds at stated concentrations, and incubated at 37 °C and 5% CO_2 for 24 h. Cells were then examined for GFP expression using a BD FACSCelesta flow cytometer. In a subset of experiments, cells were fixed and stained for intracellular $p24^{Gag}$ (KC57-RD1 antibody) and analysed by flow cytometry as described previously (Schonhofer et al., 2021). The previously described inhibitor apigenin (10 μM) was used as a positive control (Schonhofer et al., 2021).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2021.112887.

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