Efficient synthesis of \((R)\)-harmonine – the toxic principle of the multicolored Asian lady beetle \((Harmonia axyridis)\)†

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A flexible synthetic route to \((R)\)-harmonine \((\text{\textcopyright})-1\), the toxic principle of the Asian lady beetle \(Harmonia axyridis\) \((H. axyridis)\), via reductive olefination of the macrocyclic lactone \((\text{\textcopyright})-5\), is reported. High enantio-meric purity is achieved by enantioselective saponification of the lactone \(\text{rac}-5\) with horse liver esterase. Minor modifications in the synthetic route give access to racemic and chiral harmonine \((\text{\textcopyright})\), analogs and putative biosynthetic precursors. In addition, the antimicrobial activity of harmonine against \(Leishmania major\) \((L. major)\) is demonstrated and provides the rationale for harmonine-based drug development against parasitic diseases.

Introduction

The multicolored Asian lady beetle \(H. axyridis\), also known as the harlequin ladybird, is natively distributed from southern Siberia to southern China and from the Altai mountains to the Pacific coast.\(^1\) This tree dwelling beetle, of the family \(Coccinellidae\), is an important predator of aphids and scale insects and has been introduced for biological control in many countries. Over time populations began to establish and two decades ago \(H. axyridis\) became an invasive species in North America, Europe and South America threatening the native lady beetles.\(^2\)–\(^3\) The invasive success arises from intra-guild predation\(^2\) and from \(H. axyridis\)' resistance to pathogenicity.\(^7\) When a lady beetle is disturbed or attacked it releases droplets of hemolymph from the tibio-femoral joints of its legs. This behavior is referred to as reflex-bleeding.\(^4\)–\(^5\) The repellent and sometimes toxic properties of the hemolymph are due to some alkaloids, which are considered to be synthesized \textit{de novo} by the beetles.\(^8\) \(H. axyridis\) produces \((R)\)-harmonine \((\text{\textcopyright})-1\) \((17R,9Z)\)-octadec-9-ene-1,17-diamine) as the major defense compound \((\text{\textcopyright})\).

\((R)\)-Harmonine \((\text{\textcopyright})-1\) displays antibacterial activity against fast-growing mycobacteria, \(Mycobacterium tuberculosis\), and \(Plasmodium falciparum\) \((P. falciparum)\), demonstrates multi-stage antimalarial activity,\(^7\) and exhibits cytotoxicity against human tumor cell lines.\(^8\) Recently microsporidia from the hemolymph of \(H. axyridis\) were shown to infect intra-guild predators.\(^9\) In this context, \((R)\)-harmonine \((\text{\textcopyright})-1\) was postulated to protect the harlequin beetle against self-infection.\(^10\) Currently, harmonine \((\text{\textcopyright})-1\) is considered a promising lead for clinical and agricultural use (yellow biotechnology).\(^11\) Although harmonine \((\text{\textcopyright})-1\) has been isolated\(^12\) and synthesized previously,\(^12\)–\(^14\) there is a need for rapid and efficient syntheses of harmonine \((\text{\textcopyright})-1\) and related molecules, since the mode of action is still unknown. In particular, for bio-assays larger quantities are needed.

\(L. major\) is the causative agent of cutaneous leishmaniasis with an estimated annual incidence of 800 000–1.3 million new infections worldwide.\(^15,16\) Current chemotherapy against leishmaniasis is limited due to the continuous development of drug resistance accompanied by severe side effects.\(^17\) Therefore, naturally derived compounds like harmonine are investigated to identify and develop new therapies against leishmaniasis. The antiparasitic activity of harmonine against \(P. falciparum\) has already been reported and prompted the assessment of its activity against \(L. major\).\(^7\)

Here, we report a short and flexible synthetic route to harmonine \((\text{\textcopyright})-1\) in only a few steps starting from the readily synthesizable macrocyclic lactone \((\text{\textcopyright})-5\) \((\text{\textcopyright})-5\) with horse liver esterase.

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available macrocyclic lactone (S)-5. One-pot olefination of (S)-5 directly provides the basic skeleton of harmonine ((R)-1) and allows the synthesis of biosynthetic precursors and structural analogs via functional group modifications. Furthermore, we report the antiparasitic activity of harmonine against the causative agent of cutaneous leishmaniasis, *L. major*. Cultivation in the presence of (R)-1 leads to the inhibition of parasitic proliferation with a consequent early necrotic cell death phenotype.

**Results and discussion**

According to Scheme 1 the harmonine backbone can be obtained from the lactone (S)-5 and the phosphonium salt 4 by a Wittig-type reaction. The chiral lactone (S)-5 is available with high ee and excellent yield by enantioselective hydrolysis with horse liver esterase. Furthermore, synthetic intermediates like ((S)-3) are promising candidates for biosynthetic and pharmaceutical studies. By modifying the chain length of the phosphonium salt or the ring size of the lactone, analogs with different positions of the double bond and molecular size become available, thus opening a new synthetic route to harmonine-like compounds using a unified procedure.

Following the retrosynthetic strategy the backbone of harmonine (rac-1) was assembled by reductive olefination from rac-5 and the ylide of (9-isopropoxy-9-oxononyl)triphenylphosphonium bromide (4) in a single operation. The resulting isopropylester rac-6 was converted into the amide rac-8 or directly reduced to the diol rac-3 (Scheme 2). The lactone rac-5 was obtained from commercial cyclooctanone and the phosphonium salt 4 from 9-bromononanoic acid after esterification and reaction with triphenylphosphine.

The one-pot “reductive olefination” generates from the lactone rac-5 and DiBAlH at low temperatures an organo aluminium acetal that decomposes above −40 °C to a hydroxy aldehyde which reacts in situ with the phosphorane to the hydroxyester rac-6 ((Z)/(E) > 98/2). The isopropylester was used to minimize side reactions during the Wittig olefination. Reduction of rac-6 with lithium aluminum hydride afforded the diol rac-3 in quantitative yield. Following the Mitsunobu protocol the diol rac-3 was converted into the diazide rac-7 using triphenylphosphine (PPh3), diphenyl phosphorazidate (DPPA) and disisopropyl azodicarboxylate (DIAD). The final reduction of rac-7 with an excess of LiAlH4 afforded racemic harmonine (rac-1) in quantitative yield. Overall, racemic harmonine (rac-1) could be prepared in only four steps and 22% overall yield starting from rac-5 and 4. Spectral data of rac-1 were identical to literature values.

For biosynthetic studies and pharmaceutical assays the (Z)-18-aminoocotadec-9-en-2-ol (rac-2) was of interest. Conversion of the isopropylester rac-6 into the amide rac-8 was readily achieved using magnesium nitride in refluxing methanol (Scheme 2). Subsequent reduction with LiAlH4 provided the aminoalcohol rac-2 in nearly quantitative yields over two steps.

The synthetic protocol was easily extended to chiral (17R,9Z)-1,17-diaminoocotadec-9-ene ((R)-1). The key step was a kinetically controlled enantioselective saponification of the lactone rac-5 which left behind unreacted (S)-lactone ((S)-5) (ee > 99.5%) in 43% yield (Scheme 3). For hydrolysis the lactone (rac-5) was suspended in a NaH2PO4-buffer (0.1 M, pH = 7.2) and horse liver esterase (HLE) was added as lyophilized powder. The hydrolysis started immediately and the pH was kept constant by addition of dil. NaOH (0.5 M) to avoid spontaneous hydrolysis.

![Scheme 1 Retrosynthesis of harmonine ((R)-1).](image)

![Scheme 2 Synthetic sequence to harmonine (rac-1) and a putative biosynthetic precursor rac-2.](image)
The progress of the reaction was monitored using a GC-MS equipped with a chiral column (β-6TBDM) for resolution of the enantiomers (Fig. 2).

After hydrolysis of the (R)-lactone, the reaction stopped and both the remaining lactone (S)-5 and the hydroxycid (R)-9 were obtained with good yield and excellent ee (S)-5 (>99.5%) and (R)-9 (>99%).

The chiral lactone (S)-5 was converted into (R)-harmonine ((R)-1) using the same sequence of reactions as shown in Scheme 2. The spectral data of (R)-1 were identical to literature values.\(^8,12,13\)

The ee of (R)-harmonine ((R)-1) was determined by \(^19\)F NMR using (S)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA chloride) for derivatization.\(^27,28\) Due to the large chemical shifts of the α-CF\(_3\) groups of the MTPA-diastereomers the high ee of (R)-1 was reliably confirmed (ee > 97%).

For the evaluation of the antileishmanial activity of harmonine, the AlamarBlue® assay with \(L.\) major promastigotes and the Britelite™ assay with Luciferase-transgenic \(L.\) major amastigotes were employed (Table 1). The cytotoxic effect of harmonine against host cells was investigated in bone marrow-derived macrophages (BMDM). The half maximal inhibitory concentration (IC\(_{50}\)) of harmonine was detected at 14.2 µM against \(L.\) major promastigotes and at 2.4 µM against the intra-

Table 1  Antileishmanial activity of harmonine

|                | \(L.\) major promastigotes | \(L.\) major amastigotes | BMDM\(^b\) | SI\(^c\) |
|----------------|-----------------------------|---------------------------|-------------|---------|
| \((R)\)-Harmonine | 14.2\(^a\)                  | 2.4                       | 36.5        | 15.2    |
| Miltefosine    | 36.2                        | 33.0                      | 63.5        | 2.0     |

\(^a\) IC\(_{50}\): half maximal inhibitory concentration. \(^b\) BMDM: bone marrow-derived macrophages. \(^c\) SI (Selectivity Index): IC\(_{50}\) for BMDM IC\(_{50}\) for \(L.\) major. \(^d\) IC\(_{50}\) synthetic rac-harmonine: 13.2.

cellular and clinically relevant \(L.\) major amastigote form. When compared to Miltefosine (1-hexadecylphosphocholine, positive control) with an IC\(_{50}\) value of 36.2 µM against \(L.\) major promastigotes and 33.0 µM against \(L.\) major amastigotes,\(^29\) harmonine showed leishmanicidal activities at significantly lower concentrations. Miltefosine showed an IC\(_{50}\) value of 65.5 µM and harmonine showed a value of 36.5 µM against BMDM. The antileishmanial efficacy of a tested drug compared to its cytotoxicity against host cells is defined as the selectivity index (SI). SIs > 20 are considered excellent, as high antileishmanial activity and low cytotoxicity are desirable prerequisites for drug development.\(^30\) Here, harmonine shows a very good SI of 15.2 towards \(L.\) major which is considerably higher than the SI of 2.0 of Miltefosine.

The antileishmanial effect of harmonine as shown in Table 1 was further investigated. The cell morphology of \(L.\) major promastigotes was studied by means of transmitted light microscopy upon treatment with harmonine for 6 h, 10 h, and 24 h (Fig. 3). The visual examination of promastigotes incubated with harmonine showed rapid changes of the cell morphology. After 24 h only dead cells were observed upon harmonine-treatment whereas in the presence of dimethyl sulfoxide (DMSO) no significant changes in the cell morphology of the parasites were observed. Rounding of cells upon harmonine treatment was induced after 6 h of culture and dead cells were visible after 10 h of cultivation. DMSO-treated control \(L.\) major promastigotes show upon 6 h and 10 h of culture the characteristic flagellated and slender shape of the viable and unaffected parasite (Fig. 3).

The type of cell death induced by harmonine was investigated using flow cytometric approaches. The loss of membrane integrity in necrotic cells and the translocation of phosphatidylserine (PS) to the outside of the cellular membrane of apoptotic cells can be determined by Annexin V (AV, binds PS) and PI (DNA-binding) staining.\(^31\) Double staining with AV-fluorescein isothiocyanate (FITC) and PI allows the discrimination between four \textit{Leishmania} cell death phenotypes as described elsewhere: live (AV\(^{-}\)/PI\(^{-}\)), late necrotic/late apoptotic (AV\(^{+}\)/PI\(^{-}\)), early necrotic (AV\(^{-}\)/PI\(^{+}\)), and early apoptotic cells (AV\(^{+}\)/PI\(^{+}\)).\(^31,32\)

Harmonine-treatment for 24 h induced early necrotic cell death in 31.6% and late necrotic/late apoptotic cell death in 51.2% of all the treated cells (Fig. 3). This means that after 24 h of harmonine-treatment a total of 82.8% of cells were
as is reported elsewhere,7 and the activity against *L. major* (Fig. 3). The increase of late necrotic/late apoptotic cells to 51.15% after 24 h is a consequence of an early necrotic cell death phenotype, as the rupture of the cell membrane allows AV to bind to PS in the dead parasite.

### Conclusion

In conclusion a highly efficient and flexible synthetic route to chiral (R)-harmonine ((R)-1) is reported. A highly enantioselective hydrolysis of rac-5 affords both the remaining lactone (S)-5 and the hydroxy acid (R)-9 in high yield and excellent enantiomeric purities. Reductive olefination of the lactone (S)-5 with readily available phosphonium ylides gives direct access to the backbone of harmonine ((R)-1). Subsequent functional group modification provides derivatives and analogs of the natural product for structure-activity studies and mechanistic analyses to unravel the mode of action of the ladybeetle alkaloid harmonine ((R)-1). The pronounced activity of harmonine ((R)-1) against mycobacteria or the malaria parasite *P. falciparum* as is reported elsewhere,7 and the activity against *L. major* as is shown for the first time in the present study are already encouraging observations for further investigations. Readily available synthetic harmonine and analogs may provide a base for the development of novel anti-parasitic drugs with both parasitocidal and transmission-blocking activities.7

### Experimental

#### Synthesis

**General methods.** NMR spectra were recorded using a Bruker AV 400 spectrometer (Bruker, Rheinstetten/Karlsruhe, Germany) operating at 400 MHz (1H), 100 MHz (13C) and 376.5 MHz (19F). Chemical shifts (δ) in 1H, 13C and 19F NMR are given in ppm and are referenced to the residual solvent peak (CDCl3: 7.27 (1H NMR); 77.0 (13C NMR)). GC-MS analysis was carried out using a Trace MS, 2000 Series (ThermoQuest, Engelsbach, Germany) equipped with an Alltech DB5 column (30 m × 0.25 m, 0.25 µm); carrier gas: helium. Enantiomers were separated on a Hydrodex-β-6TBDM column (25 m × 0.25 m) and analyzed with an ITQ 900 (Thermo Fischer Scientific, Bremen, Germany). HPLC-MS spectra were recorded using a Finnigan LTQ (Thermo Electron Company, Waltham, Massachusetts, USA) MS in APCI mode and a Purospher STAR RP18 column (250 mm × 2 mm, 5 µm). Infrared spectra were recorded using a Bruker Equinox 55 FTIR spectrophotometer over the 700–4000 cm⁻¹ range with a spectral resolution of 2 cm⁻¹ using the transmission mode. The optical rotation was measured with a JASCO P1030 polarimeter at 22–24 °C at a specific wavelength of 589 nm. High resolution mass spectra (HR-MS) were either obtained using a Hewlett Packard 6890 GC operated with a Phenomenex ZBSHT column (30 m × 0.25 m, 0.25 µm) and connected to a Massspec MS02 (Micromass, UK) (EI, 70 eV) or a QExactive Plus MS (Thermo Fischer Scientific, Bremen, Germany) equipped with an RP-18 column (150 mm × 2.1 mm, 120 Å) or via direct injection into the ESI source (capillary temp.: 275 °C). For preparative column chromatography silica gel (40–63 µm, Macherey-Nagel, Düren,
Germany) or Lichroprep RP-18 silica gel (40–63 μm, Merck, Darmstadt, Germany) was used. Thin layer chromatography was conducted on silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany). The compounds were detected using Hanessian’s stain, vanillin or potassium permanganate stain. Except for diethyl ether all solvents purchased were of HPLC grade and used without further purification. Diethyl ether was distilled prior to use. Anhydrous solvents were purchased as such. Horse liver esterase was obtained as lyophilized powder (0.5–1.0 U mg⁻¹, Sigma Aldrich, Saint Louis, MO, USA).

**Isopropyl (9Z)-17-hydroxyoctadec-9-enoate (rac-6).** A cold solution (−78 °C) of 9-methyloxan-2-one (rac-5) (78 mg, 0.5 mmol) in dry toluene (2.7 ml) was treated within 10 min with DiBAIH (0.6 ml, 0.6 mmol, 1 M in hexane). Stirring was continued for an additional 40 minutes and the mixture was hydrolyzed by addition of 5% HCl (2.6 ml). The layers were separated and the aqueous phase was extracted with Et₂O (2×5 ml). The combined organic extracts were washed with 5% HCl (2.6 ml) and cooled to 0 °C. PPh₃ (836 mg, 3.19 mmol), DPPA (340.2985; IR (thin film, cm⁻¹) ν 3437 (br, m), 2977 (m), 2964 (s); 1H NMR (400 MHz, CDCl₃) δ 5.40 (s), 1.06, CHCl₃). All other spectral data were identical to rac-6.}

**MRMS m/z calc for C₃₄H₆₄NaO₂ 340.2978 [M⁺], found 340.2985; IR (thin film, cm⁻¹) ν 3437 (br, m), 2977 (m), 2964 (m), 2928 (s), 2855 (s), 1733 (s), 1655 (w), 1464 (m), 1374 (m), 1249 (m), 1145 (m), 1110 (s); 1H NMR (400 MHz, CDCl₃) δ 5.40–5.31 (m, 2 H, H-10/H-9), 5.01 (sp, 3J₁,2 = 3.7 Hz, 2 H, H-2), 1.49–1.38 (m, 2 H, H-16), 1.36–1.27 (m, 18 H, H-14), 1.23 (d, 3J₁,2 = 6.3 Hz, 6 H, H-2′,H-3′), 1.19 (d, 3J₁,2 = 6.3 Hz, 2 H, H-18), 1.19 (d, 3J₁,2 = 6.3 Hz, 2 H, H-17)).

**13C NMR (100 MHz, CDCl₃) δ 175.4 (C-1′), 129.8 (C-10/C-9), 68.1 (C-17), 67.3 (C-18), 29.7, 29.5, 29.4, 29.2, 29.1 (C-14–C-12/C-7–C-4), 27.1 (C-11–C-8), 25.7 (C-15/C-3), 23.4 (C-18), 21.8 (C-2′/C-2′′).

**17(5Z,9Z)-17-Octadecyl-9-enoate (rac-6).** It is prepared from (9Z)-9-methyloxan-2-one ((S)-5) (750 mg, 4.80 mmol) as described. After purification, (S)-5 was obtained as a colorless oil (366 mg, 1.08 mmol, 23%). [α]D²⁰ +2.9 (c 1.00, CHCl₃). All other spectral data were identical to rac-6.

**17(5Z,9Z)-17-Octadecyl-9-enoate (rac-6).** LiAlH₄ (99 mg, 2.62 mmol) was suspended at 0 °C in dry THF (7.5 ml) and the ester rac-6 (297 mg, 0.87 mmol) was added slowly. The reaction mixture was allowed to come to room temperature and stirred for 4.5 hours. Et₂O (9 ml) and water (10 ml) were added, the phases were separated and the aqueous layer was extracted with Et₂O (3 × 10 ml). The combined organic extracts were washed with water (30 ml) and dried over Na₂SO₄. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel using hexane-ethyl acetate (1:1) for elution. After removal of the solvents, rac-3 was obtained as a slightly yellow oil (243 mg, 0.85 mmol, 98%). Rₚ = 0.35 (hexane–ethyl acetate 1:1).

**MRMS m/z calc for C₁₈H₃₆O₂ 284.2715 [M⁺], found 284.2705; IR (thin film, cm⁻¹) ν 3332 (br, m), 3004 (w), 2926 (s), 2854 (s), 1645 (w), 1373 (m); 1H NMR (400 MHz, CDCl₃) δ 5.40–5.30 (m, 2 H, H-10/H-9), 3.79 (tq, 3J₁,2 = 3.7 Hz, 2 H, H-2), 1.49–1.38 (m, 2 H, H-16), 1.38–1.25 (m, 18 H, H-14–H-12/H-7–H-4), 1.18 (d, 3J₁,2 = 6.0 Hz, 3 H, H-18), 1.17 (d, 3J₁,2 = 6.0 Hz, 3 H, H-18). 13C NMR (100 MHz, CDCl₃) δ 129.9, 129.8 (C-10/C-9), 68.2 (C-17), 63.0 (C-1), 39.3 (C-16), 32.7 (C-2), 29.7, 29.5, 29.4, 29.2, 29.1 (C-14–C-12/C-7–C-4), 27.1 (C-11–C-8), 25.7 (C-15/C-3), 23.4 (C-18).

**17(5Z,9Z)-17-Octadecyl-9-enoate (rac-6).** (R)-7. It is prepared from (17S,9S)-17-hydroxyoctadec-9-enoate (S)-5 (295 mg, 0.87 mmol). After column chromatography (S)-3 was obtained as a colorless oil (245 mg, 0.86 mmol, 98%). [α]D²⁰ +3.8 (c 1.00, CHCl₃). All other spectral data were identical to rac-3.

**17(5Z,9Z)-17-Octadecyl-9-enoate (rac-6).** (R)-7. (9Z)-Octadecyl-3-ene-1,17-diol (rac-1). LiAlH₄ (177 mg, 3.95 mmol) was suspended with stirring in dry THF (11 ml) and cooled to 0 °C. A solution of (9Z)-17-diazidooctadec-9-ene (rac-7) (165 mg, 0.49 mmol) in dry THF (5 ml) was added...
dropwise and the suspension was warmed to room temperature. After stirring for 4 hours, water (30 ml) and CHCl3 (30 ml) were added, the layers were separated and the aqueous phase was extracted with CHCl3 (6 x 30 ml). The organic extracts were combined and solvents were removed under reduced pressure. The product was partitioned between MTBE (80 ml) and 2% HCl (80 ml). The layers were separated, the aqueous phase was carefully extracted with MTBE (3 x 20 ml) and the combined organic extracts were washed with 2% HCl (30 ml). The aqueous solutions were combined and MTBE (40 ml) and NH4 (25% aqueous solution, 80 ml) were added. After separation of the two layers, the aqueous phase was extracted with MTBE (3 x 60 ml). The combined organic extracts were dried over Na2SO4 and the solvent was evaporated under reduced pressure to yield rac-1 as a colorless oil (139 mg, 0.49 mmol, quant.).

HRMS m/z calc for C18H39N2 283.3108 [M + H]+, found 283.3106; IR (thin film, cm−1) ν 3332 (br, m), 3004 (m), 2921 (s), 2851 (s), 1639 (w), 1468 (m), 1456 (m), 1390 (m), 1331 (m); 1H NMR (400 MHz, CDCl3) δ 5.38–5.27 (m, 2 H, H-10/H-9), 2.85 (tq, J7,16 = 6.0 Hz, 1 H, H-17), 2.66 (t, J6,5 = 7.0 Hz, 2 H, H-2), 2.04–1.91 (m, 4 H, H-11/H-8), 1.50–1.38 (m, 6 H, H-2/H-3), 1.36–1.21 (m, 20 H, H-16), 1.31–1.26 (m, 20 H, H-14b/H-15a), 1.23 (m, 4 H, H-15H-13). 13C NMR (100 MHz, CDCl3) δ 129.8, 129.8 (C10/C9), 46.9 (C17), 42.1 (C11), 40.1 (C16), 33.7 (C2), 29.7, 29.6, 29.6, 29.4, 29.4, 29.2, 29.2 (C14-C12/C7-C4), 27.1 (C11/C8), 26.8 (C3), 26.3 (C15), 23.8 (C-18).

(17R,9Z)-Octadec-9-ene-1,17-diamine ((R)-1) (harminone). It is prepared from (17R,9Z)-1,17-diazidoctadec-9-ene ((R)-7) (161 mg, 0.48 mmol). Harminone ((R)-1) was obtained as a slightly yellow oil (135 mg, 0.48 mmol, 99%, ee > 97%). [α]24D −3.4 (c 1.04, CHCl3). Spectroscopic data were identical to rac-1.

HRMS m/z calc for C18H38NO 284.2948 [M + H]+, found 284.2935; IR (thin film, cm−1) ν 3332 (br, m), 3004 (m), 2925 (s), 2853 (s), 1632 (m), 1572 (m), 1464 (m), 1372 (m), 1315 (s); 1H NMR (400 MHz, CDCl3) δ 5.38–5.28 (m, 2 H, H-10/H-9), 3.75 (tq, J7,16 = 6.1 Hz, 1 H, H-17), 2.66 (t, J6,5 = 7.2 Hz, 2 H, H-2), 2.04–1.91 (m, 4 H, H-11/H-8), 1.82–1.76 (m, 3 H, N-H2, O-H2), 1.48–1.35 (m, 5 H, H-16/H-15a/H-2), 1.35–1.23 (m, 17 H, H-15b/H-14–H-12/H-7–H-3), 1.15 (d, J3,2 = 6.1 Hz, 3 H, H-18). 13C NMR (100 MHz, CDCl3) δ 130.3, 130.2 (C10/C9–E), 129.8, 129.8 (C10/C9), 67.7 (C17–E), 67.7 (C17–E), 42.1 (C11), 33.6 (C2–E), 33.6 (C2–E), 32.5 (C11/C8–E), 29.6, 29.6, 29.5, 29.4, 29.4, 29.2, 29.1, 25.7 (C14-C12/C7-C4), 27.1 (C11/C8), 26.8 (C3), 26.8 (C3), 25.7 (C-15), 23.4 (C-18).

Enzymatic hydrolysis of lactone (rac-5)

Racemic 9-methyloxonan-2-one (rac-5) (3.50 g, 22.4 mmol) was suspended in NaH2PO4 buffer (0.1 M, pH = 7.2, 100 ml) and stirred for 15 minutes. Then horse liver esterase (350 mg, lyophilized powder) was added and the pH was kept constant by dropwise addition of 0.5 M NaOH during the whole reaction time. After 12 hours, another portion of horse liver esterase (70 mg) was added and stirring was continued for 4 hours. Then Celite (3.50 g) and ice (7.00 g) were added, the suspension was stirred for 5 minutes and the solids were filtered off.
The filter cake was washed with Et₂O (2 × 50 ml). The layers of the filtrate were separated and the aqueous phase was extracted with Et₂O (3 × 50 ml). The combined organic extracts were washed with a saturated NaHCO₃ solution (100 ml) and dried over Na₂SO₄. Evaporation of the solvent yielded (9S)-9-methyloxan-2-one ((S)-9) as a colorless liquid.

(9S)-9-Methyl-oxan-2-one ((S)-9). Yield: 1.51 g (9.67 mmol, 86% of (S)-enantiomer, ee ≥ 99.5%). [α] D 23 +38.1 (c 1.00, THF).

HRMS m/z calculated for C₉H₁₆O₂Na 197.1148 [M + Na]⁺, found 197.1146; IR (thin film, cm⁻¹) ν 2937 (s), 2857 (s), 1559 (s), 1473 (s), 1384 (s), 1162 (s), 1103 (m); ¹H NMR (400 MHz, CDCl₃) δ 5.03 (m, 1 H, H-8), 2.25 (m, 2 H, H-2), 1.93 (m, 1 H, H-4a), 1.82–1.74 (m, 1 H, H-7a), 1.72–1.64 (m, 1 H, H-3a), 1.67–1.55 (m, 2 H, H-6a/H-3b), 1.55–1.43 (m, 3 H, H-7b/H-5a/H-4b), 1.37–1.27 (m, 2 H, H-6b/H-5b), 1.25 (d, J ₃,₂ = 6.5 Hz, 3 H, H-9); ¹³C NMR (100 MHz, CDCl₃) δ 175.1 (C-1), 71.5 (C-8), 35.7 (C-2), 35.1 (C-7), 29.4 (C-5), 25.0 (C-4), 23.9 (C-3), 21.8 (C-2), 20.9 (C-1).

(8R)-8-Hydroxyoxanonic acid ((R)-9). The water phase was covered with Et₂O (100 ml) and 2 M HCl (60 ml) was added. The layers were separated and the aqueous phase was extracted with Et₂O (3 × 100 ml). The combined organic extracts were washed with a saturated NaCl solution (150 ml) and dried over Na₂SO₄. The solvent was removed under reduced pressure to yield (8R)-8-hydroxyoxanonic acid ((R)-9) as colorless wax (1.52 g, 8.72 mmol, 78% of (R)-enantiomer, ee ≥ 99%). [α] D 23 −8.5 (c 1.00, THF).

HRMS m/z calculated for C₉H₁₆O₂Na 197.1148 [M + Na]⁺, found 197.1146; IR (thin film, cm⁻¹) ν 3391 (br, s), 2933 (s), 2858 (s), 1731 (s), 1466 (m), 1368 (m), 1282 (s), 1103 (m); ¹H NMR (400 MHz, CDCl₃) δ 7.67 (m, Har-5/Har-3), 4.96 (sp, 3 J ₂,₁  = 6.2 Hz, 1 H, H-1′), 3.82–3.71 (m, 2 H, H-9), 2.20 (t, J ₂,₃ = 7.5 Hz, 1 H, H-2), 1.68–1.56 (m, 4 H, H-8/H-7), 1.53 (m, 2 H, H-3), 1.30–1.21 (m, 6 H, H-6/H-4), 1.20 (d, J ₂,₃ = 6.2 Hz, 6 H, H-2″/H-2′); ¹³C NMR (100 MHz, CDCl₃) δ 173.3 (C-1), 135.0, 135.0 (Car-4), 133.7, 133.6 (Car-6/Car-2), 131.3, 135.0 (Car-5/Car-3), 118.8, 118.0 (Car-1), 67.3 (C-1′), 34.6 (C-2′), 30.3, 30.2 (C-7), 28.9, 28.9, 28.7 (C-6/C-4), 24.8 (C-3′), 23.0, 22.6 (C-9), 22.6, 22.5 (C-8), 21.8 (C-2′/C-2″).

Antileishmanial activity

The virulent L. major isolate (MHOM/IL/81/FE/BNI) and L. major promastigotes were maintained by continuous passage in female BALB/c mice. L. major amastigotes were isolated from lesions as described previously. Isopropyl 9-bromonononoate (10) was obtained as a colorless oil (5.23 g, 21.92 mmol) in dry 2-propanol (50 ml) and sulfuric acid (1 ml) was added. The flask was equipped with Dean-Stark apparatus and the solution was heated to reflux for 19 hours. After the solution was cooled down to room temperature, 2-propanol was removed under reduced pressure, the residue was dissolved in Et₂O (20 ml) and the organic solution was washed with a saturated Na₂CO₃ solution (20 ml), distilled water (20 ml) and a saturated NaCl solution (20 ml). The ethereal solution was dried over MgSO₄, the solvent was removed and the crude product was purified by column chromatography on silica gel (DCM). After removal of the solvent, 10 was obtained as a colorless oil (5.23 g, 18.73 mmol, 86%). Rf = 0.70 (DCM).

HRMS m/z calculated for C₁₃H₁₃O₂BrNa, C₁₃H₁₂O₂¹⁸BrNa 301.0774, 303.0753 [M + Na]⁺, found 301.0776, 303.0753; IR (thin film, cm⁻¹) ν 2979 (m), 2932 (s), 2857 (m), 1731 (s), 1466 (m), 1374 (m), 1256 (m), 1181 (m), 1145 (w), 964 (w); ¹H NMR (400 MHz, CDCl₃) δ 5.00 (sp, J ₂,₁  = 6.2 Hz, 1 H, H-1′), 3.40 (t, J ₉,₈ = 6.9 Hz, 2 H, H-9), 2.26 (t, J ₂,₃ = 7.6 Hz, 2 H, H-2), 1.85 (m, 2 H, H-8), 1.61 (m, 2 H, H-3), 1.42 (m, 2 H, H-7), 1.34–1.28 (m, 6 H, H-6/H-4), 1.22 (d, J ₂,₃ = 6.2 Hz, 6 H, H-2″/H-2′); ¹³C NMR (100 MHz, CDCl₃) δ 173.3 (C-1), 67.3 (C-1′), 34.6 (C-2′), 30.3 (C-7), 28.9, 28.9, 28.7 (C-6/C-4), 24.8 (C-3′), 23.0, 22.6 (C-9), 22.6, 22.5 (C-8), 21.8 (C-2′/C-2″).

Diff-Quick staining for transmitted light microscopy

After incubation for 6 h, 10 h, and 24 h in the presence of 30 μM harmane or 1% DMSO as the solvent control, L. major promastigotes were harvested and centrifuged using a CytoSpin 3 centrifuge (Shandon, Frankfurt, Germany) on microscopic slides. Cytoplasmic preparations were stained using the Differential Quick stain (Diff-Quick) dye (Medion Diagnostics AG, Duedingen, Switzerland), according to the manufacturer’s protocol. Diff-Quick stains the leishmanial nuclei, the kinetoplast dark purple and the cytoplasm light purple allowing the
observation of phenotypic changes within the parasite. The stained cells were analyzed by transmitted light microscopy under a 50× objective on a Nikon ECLIPSE 50i microscope equipped with a digital camera (Nikon, Tokyo, Japan). The images were processed using NIS Elements D software (Nikon).

**Determination of the cell death phenotype by flow cytometric analysis**

*L. major* promastigotes were either treated for 6 h, 10 h, and 24 h with 30 µM harmol or 1% DMSO as the solvent control. Cell staining was performed using an Annexin V-FITC Apoptosis detection kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer’s protocol. The stained samples were immediately analyzed by flow cytometry using a MACS Quant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany).

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