Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4140–4143

(2*R*,3*S*)-(+)- and (2*S*,3*R*)-(−)-Halofuginone lactate: Synthesis, absolute configuration, and activity against *Cryptosporidium parvum*

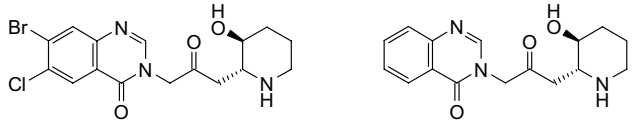
Michael R. Linder,[a] Anja R. Heckeroth,[a] Michael Najdrowski,[b] Arwid Daugschies,[b] Dieter Schollmeyer[c] and Christian Miculka[a,*]

[a]*Intervet Innovation GmbH, Zur Propstei, 55270 Schwabenheim, Germany*
[b]*Institute of Parasitology, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 35, 04103 Leipzig, Germany*
[c]*Institute of Organic Chemistry, University Mainz, Duesbergweg 10-14, 55099 Mainz, Germany*

Received 30 March 2007; revised 15 May 2007; accepted 17 May 2007
Available online 21 May 2007

Abstract—The trans-enantiomers of the commercially important anti-protozoal compound Halofuginone have been prepared and characterized, and the absolute configuration was assigned by X-ray crystallography. The activity of both enantiomers against *Cryptosporidium parvum* was determined in vitro and related to acute toxicity in vivo. It was shown that both the activity and the toxicity are properties of the (2*R*,3*S*)-enantiomer. We conclude that with respect to broadening the therapeutic window there is no advantage in application of one enantiomer over the application of the racemic mixture in the treatment of *C. parvum* infections.

Racemic Halofuginone [(*rac*)-**1**] as lactate is used in the prevention and treatment of diarrhea due to *Cryptosporidium parvum* in non-ruminating calves (Halocur®), whereas the hydrobromide salt is applied as an anticoccidial feed additive for broilers and for turkeys (Stenorol®). The potential of (*rac*)-**1** in human applications relying on its type specific inhibition of collagen synthesis like treatment of fibrosis[1] and uterine fibroids[2] has been explored. The therapeutic window of the application in calves is narrow, as serious side-effects occur already at twice the therapeutic dose.[3] (*rac*)-**1** has trans-configuration on the piperidine moiety, and is structurally related to the natural compound Febrifugine (**2**), the absolute configuration of which was originally described as being (2*S*,3*R*), later to be corrected by Kobayashi[4] to be its optical antipode (2*R*,3*S*) (Fig. 1). In the same paper,[4] the antimalarial activities in vitro and cytotoxicities of **2** and its optical antipode were described: The EC_{50} of naturally occurring **2** against *Plasmodium falciparum* was shown to be approximately 10^4 times lower than for its antipode, while the cytotoxicity EC_{50} dropped by a factor of 10^2.



(*rac*)-**1** (relative stereochemistry) **2** (absolute stereochemistry)

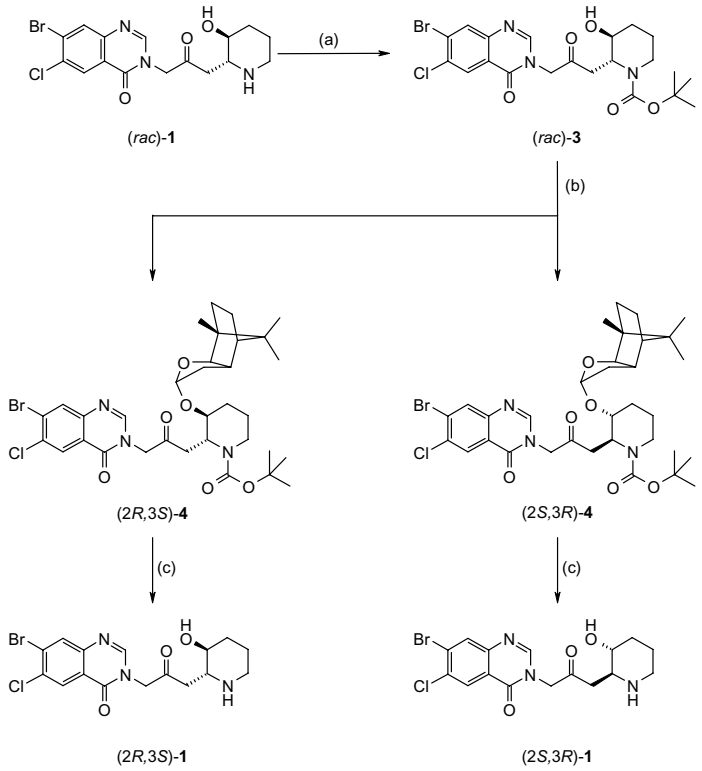
Figure 1. Halofuginone [(*rac*)-**1**] and Febrifugine (**2**).

In the context of the strategy of chiral switches[5] we investigated whether we could broaden the therapeutic window for the calf application by switching from the use of (*rac*)-**1** to a trans-configurated eutomer. Jolly et al.[6] had described the resolution of (*rac*)-**1** by addition of salts and the coccidiostatic properties of the enantiomers in poultry, but the assignment of absolute configuration had remained open. Recently, a determination of absolute configuration by synthesis was presented by Oalmann et al.[7] In this paper, we describe the separation of the (*rac*)-**1** via diastereoisomers, the determination of their absolute configuration and its relation to **2**, and the determination of the activity of both enantiomers against *C. parvum* in vitro and of toxicity in vivo.

(*rac*)-**1**[8] was Boc-protected in 69% yield. The *N*-Boc-protected Halofuginone [(*rac*)-**3**] was subsequently

Keywords: Anti-protozoal; Halofuginone; *Cryptosporidium parvum*; Chiral switch.
* Corresponding author. E-mail: christian.miculka@intervet.com

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.05.053



Scheme 1. Reagents and conditions: (a) Di-*tert*-butyl dicarbonate, dioxane/H_2O, NaOH, 0 °C, 2 h, 69%; (b) (+)-Noe-lactol® dimer, *p*-toluene sulfonic acid, $CHCl_3$, rt, 3 d, then separation on silica gel, PE/EE 4:1, [(2*R*,3*S*)-**4**] 16% and [(2*S*,3*R*)-**4**] 14%; (c) trifluoroacetic acid, CH_2Cl_2, rt, 16 h, [(2*R*,3*S*)-**1**] 45% and [(2*S*,3*R*)-**1**] 41%.

reacted with (+)-Noe lactol® dimer[9] in the presence of catalytic amounts of *p*-toluenesulfonic acid at rt for 3 days yielding a mixture of diastereoisomers (2*R*,3*S*)-**4** and (2*S*,3*R*)-**4** (Scheme1). Diastereoisomers (2*R*,3*S*)-**4** and (2*S*,3*R*)-**4** were separated by column chromatography on silica gel and obtained in yields of 16% [(2*R*,3*S*)-**4**] and 14% [(2*S*,3*R*)-**4**], respectively. One-step deprotection was achieved by trifluoroacetic acid in dichloromethane at rt yielding Halofuginone enantiomers (2*R*,3*S*)-**1** (45% yield) and (2*S*,3*R*)-**1** (41% yield). The optical purity of (2*R*,3*S*)-**1** and (2*S*,3*R*)-**1** (ee > 99%, respectively) was determined by separation of enantiomers by HPLC on a Chiralpak AD-H column. The lactates of (2*R*,3*S*)-**1** and (2*S*,3*R*)-**1** used for the *C. parvum* assay were synthesized by the addition of an aqueous solution of racemic lactic acid and evaporation of the solvent. The lactic acid contents [(2*R*,3*S*)-**1**: 96%, (2*S*,3*R*)-**1**: 99%)] were determined by ion chromatography.[10]

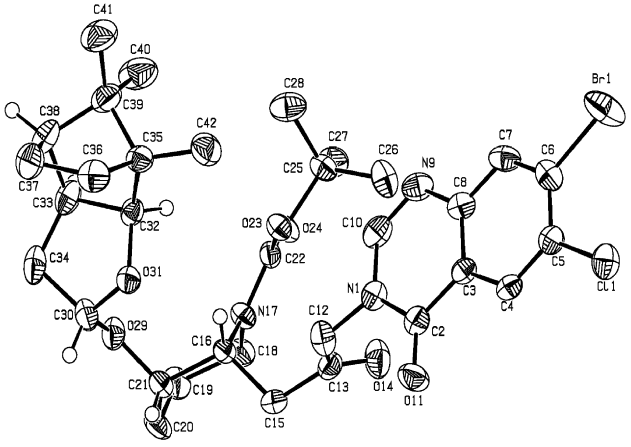


Figure 2. ORTEP view of (2*R*,3*S*)-**4** showing 50% probability displacement ellipsoids. Hydrogen atoms omitted except for atoms attached to optically active C-atoms.

Absolute stereochemical assignment was performed on the basis of the crystal structure of intermediate (2*R*,3*S*)-**4**. Figure 2 shows the X-ray structure of (2*R*,3*S*)-**4** in the crystal.[11] The absolute configuration of (2*R*,3*S*)-**4** was determined by the refinement of the Flack parameter, which showed that (2*R*,3*S*)-**4** has the following absolute configuration: C16-R, C21-S, C30-R, C32-S, C33-S, C35-R, C38-R. The geometrical parameters (bond lengths and angles) are in good agreement with commonly accepted values. Supplementary X-ray data are available online.

(*rac*)-**1** hydrobromide, (*rac*)-**1** lactate, and its enantiomers (2*R*,3*S*)-**1** lactate and (2*S*,3*R*)-**1** lactate were tested for their anticryptosporidial activity in cell cultures as described elsewhere.[12] Three of the four tested compounds exhibited a good inhibitory effect on in vitro development of *C. parvum*. (2*S*,3*R*)-**1** lactate was the least effective as it lost its efficacy already at a concentration in the range of 1–0.1 μM. Although no precise turning concentration could be determined, a steep increase in parasite development was observed with these concentrations. The measured values varied

considerably between assays, which is typical for biological assays like an in vitro determination of parasite development. For (2*R*,3*S*)-**1** lactate similar efficacy to that of a positive control monensin[12] was observed down to a concentration of 0.01 μM. A gradual increase in parasite development occurred up to about 3 nM. At higher dilutions, (2*R*,3*S*)-**1** lactate lost its activity and foci areas returned to control levels. Similar results as for (2*R*,3*S*)-**1** lactate yielded both (*rac*)-**1** lactate and (*rac*)-**1** hydrobromide. Almost the same tendency for efficacy was observed, albeit on a slightly higher level. No effect was seen at concentrations of 0.8 nM or less. The inhibitory effects of both (*rac*)-**1** hydrobromide and (*rac*)-**1** lactate, (2*R*,3*S*)-**1** lactate and (2*S*,3*R*)-**1** lactate are illustrated in Figure 3. Similar to (*rac*)-**1** lactate, the (*rac*)-**1** hydrobromide was able to exert an inhibition of about 80% up to a concentration of about 6 nM. For both compounds, the effect was apparently gone at 0.8 nM or less. With all compounds tested effective, a similar extinction of developmental stages (~80%) could be achieved, as also observed in the positive control monensin. The study confirmed (2*R*,3*S*)-**1** and (*rac*)-**1**—both as lactate and hydrobromide—to be highly effective against *C. parvum* in an in vitro assay. Supplementary efficacy data are available online.

(*rac*)-**1** lactate and its enantiomers (2*R*,3*S*)-**1** lactate and (2*S*,3*R*)-**1** lactate were tested orally for their toxic effects in 4-week-old female Crl:NMRI-mice ($n = 3$) at doses of 5, 10, and/or 20 mg/kg bodyweight. Administration of 10 mg/kg bodyweight (*rac*)-**1** lactate led to depression, that is, slow or no movement, half-closed eyes, piloerection, in one mouse after 5 h. The next day one mouse was found dead and two mice were euthanized at a moribund stage. After administration of 5 mg/kg bodyweight (*rac*)-**1** lactate all mice were depressed and trembled after 5 h. One mouse was found dead the next day, whereas the other two mice were still depressed, but did not tremble any more. Thus, the toxic effect can be assumed to be dose-dependent. While (2*R*,3*S*)-**1** lactate led to death of all mice after administration of 5 and 10 mg/kg bodyweight, the second enantiomer (2*S*,3*R*)-**1** lactate did not have an effect even at a dose of 20 mg/kg. Therefore it is concluded that only (2*R*,3*S*)-**1** lactate contributes to the toxic effect of (*rac*)-**1** lactate.

In summary, both trans-enantiomers of Halofuginone [(2*R*,3*S*)-**1** and (2*S*,3*R*)-**1**] have been prepared and characterized, including their absolute configuration, which was assigned unambiguously by X-ray crystallography. Also, the sense of optical rotation of the enantiomers was in full accordance with the sense of optical rotation of isochiral **2** (after revision of its configuration[4]) and its enantiomer. Both the activity against *C. parvum* in vitro and the toxicity in vivo of (2*R*,3*S*)-**1** lactate were significantly stronger compared to its optical antipode (2*S*,3*R*)-**1** lactate. We conclude that it is not possible to separate activity against *C. parvum* from mammalian toxicity by application of an eutomer instead of (*rac*)-**1**, as both properties are intrinsic to (2*R*,3*S*)-**1**.

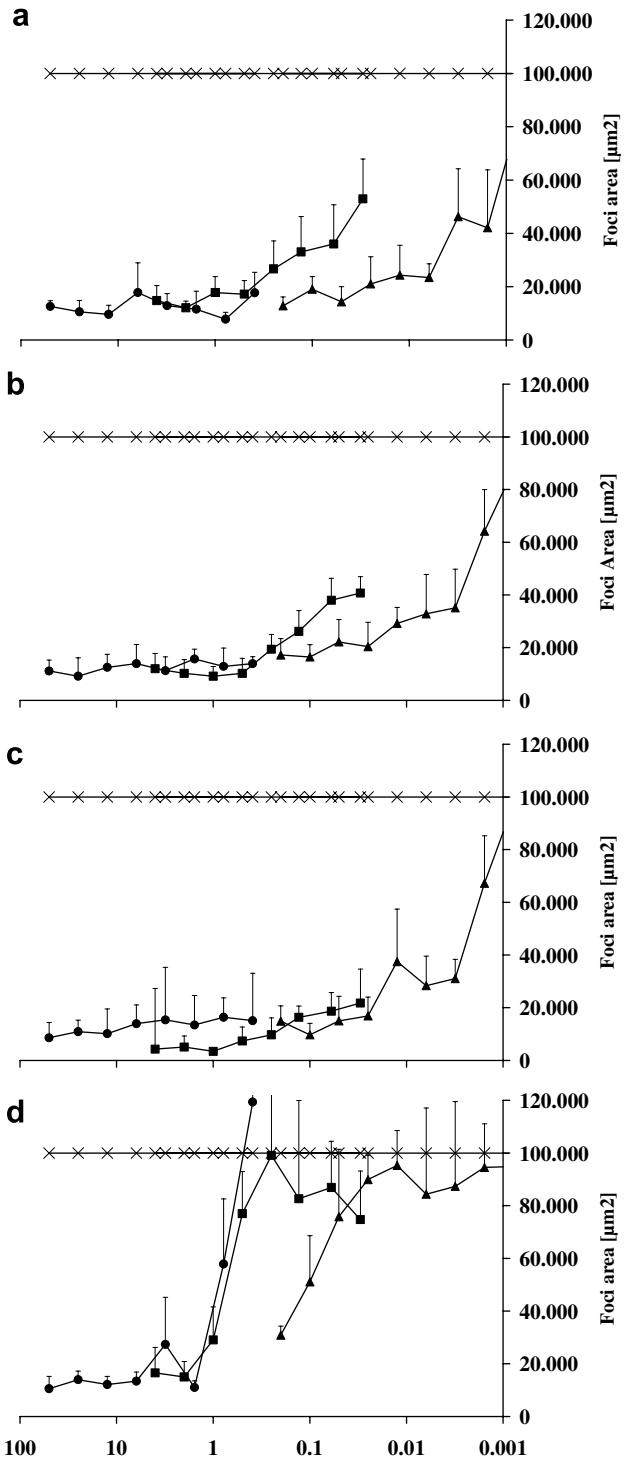


Figure 3. Effect of various concentrations of (*rac*)-**1** hydrobromide (a), (*rac*)-**1** lactate (b), (2*R*,3*S*)-**1** lactate (c), and (2*S*,3*R*)-**1** lactate (d) on the in vitro development of *C. parvum* in three subsequent dilution series (● 1st dilution series, ■ 2nd dilution series, ▲ 3rd dilution series). Control and test cultures were normalized to 100,000 μm^2 of foci area (**X**) to allow for comparison between different experiments. (2*R*,3*S*)-**1** lactate is at least 100-fold more effective than (2*S*,3*R*)-**1** lactate.

Acknowledgments

We thank M. Hielscher for the synthetic support, K.-H. Grimm for the chromatographic determination of enan-

tiomeric excesses, and W. Dindorf from the University of Mainz for elemental analyses, S. Gawlowska for the performance of in vitro *Cryptosporidium* assays, and U. Deutsch for the performance of toxicity studies.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.05.053.

References and notes

1. Pines, M.; Vlodavsky, I.; Nagler, A. *Drug Dev. Res.* **2000**, *50*, 371.
2. Nowak, R. A. *Drug Discov. Today: Therap. Strat.* **2004**, *1*, 237.
3. Peregrine, A. S. *Vet. Parasitol.* **1994**, *54*, 223.
4. Kobayashi, S.; Ueno, M.; Suzuki, R.; Ishitani, H.; Kin, H.-S.; Wataya, Y. *J. Org. Chem.* **1999**, *64*, 6833.
5. Agranat, I.; Caner, H.; Caldwell, J. *Nature Rev. Drug Disc.* **2002**, *1*, 753.
6. Jolly, J.; Rizzi, P.; Grandadam, J. A. U.S. Patent, 4,252,947, 1977.
7. Oalmann, C. J.; Richard, J.; Bockovich, N. *Abstracts of Papers*, 232nd National Meeting of the American Chemical Society, San Francisco, CA, United States, Sept 10–14, 2006; American Chemical Society: Washington, DC, 2006; ORGN-071.
8. (*rac*)-**1** was obtained from Intervet-internal sources.
9. Noe, C. R. *Chem. Ber.* **1982**, *115*, 1591.
10. Compound (*rac*)-**3**: pale brown solid. ^{1}H NMR ($CDCl_3$) 300 MHz δ 8.30 (s, 1H), 8.05 (s, 1H), 8.04 (s, 1H), 5.08 (d, J = 17.5 Hz, 1H), 4.95 (d, J = 17.5 Hz, 1H), 4.66 (m, 1H), 3.93 (s, 1H), 3.88 (s, 1H), 2.95 (t, J = 11.7 Hz, 1H), 2.80 (d. J = 4.0 Hz, 1H), 2.77 (s, 1H), 2.75–2.70 (m, 1H), 1.91–1.64 (m, 3H), 1.43 (s, 10H); ^{13}C NMR ($CDCl_3$) 75 MHz δ 199.5, 159.5, 156.3, 148.2, 146.8, 133.6, 132.5, 129.6, 127.6, 121.8, 80.7, 67.5, 54.5, 53.8, 40.7, 39.7, 28.4, 26.3, 19.0; MS (API-ES) *m/z*: 414 $[M-C_5H_9O_2+H]^+$. Compound (2*R*,3*S*)-**4**: colorless solid. ^{1}H NMR (acetone-d_6) 400 MHz δ 8.24 (s, 1H), 8.17 (s, 1H), 8.05 (s, 1H), 5.48 (d, J = 5.0 Hz, 1H), 5.20 (d, J = 18.5 Hz, 1H), 5.08 (d, J = 18.5 Hz, 1H), 4.91 (s, 1H), 4.45 (d, J = 8.1 Hz, 1H), 3.91 (d, J = 11.9 Hz, 1H), 3.74 (s, 1H), 3.00–2.78 (m, 4H), 1.90 (ddd, J_1 = 13.2 Hz, J_2 = 6.8 Hz, J_3 = 5.2 Hz, 1H), 1.78–1.47, 1.40–1.37 (2m, 9H), 1.43 (s, 9H), 1.18–1.11 (m, 1H), 1.00 (s, 3H), 0.91 (s, 3H), 0.86 (s, 3H); ^{13}C NMR (acetone-d_6) 75 MHz δ 201.1, 159.8, 156.1, 150.3, 148.7, 133.5, 133.1, 129.1, 128.1, 123.2, 109.0, 89.7, 79.8, 72.1, 54.5, 53.7, 53.1, 49.2, 48.4, 41.1, 41.0, 39.7, 33.5, 28.7, 27.3, 24.5, 21.0, 20.1, 21.2, 19.1, 15.2; MS (API-ES) *m/z*: 414 $[M-C_5H_9O_2-C_{12}H_{19}O_2+H]^+$; de > 99%; mp: 151 °C; $[\alpha]_D^{20}$ +40 (*c* 0.5, DMF). Compound (2*S*,3*R*)-**4**: ^{1}H NMR (acetone-d_6) 300 MHz δ 8.26 (s, 1H), 8.18 (s, 1H), 8.08 (s, 1H), 5.57 (d, J = 4.6 Hz, 1H), 5.15 (s, 2H), 4.93 (dd, J_1 = J_2 = 6.9 Hz, 1H), 4.24 (d, J = 9.4 Hz, 1H), 3.94 (br s, 1H), 3.74 (s, 1H), 3.06 (dd, J_1 = 15.5 Hz, J_2 = 6.7 Hz, 1H), 2.95–2.80 (m, 3H), 1.94 (m, 1H), 1.83–1.67, 1.62–1.47, 1.41–1.30 (3m, 9H), 1.45 (s, 9H), 1.20–1.11 (m, 1H), 1.00 (s, 3H), 0.94 (s, 3H), 0.85 (s, 3H); ^{13}C NMR (acetone-d_6) 75 MHz δ 201.5, 159.9, 156.3, 150.3, 148.7, 133.6, 133.2, 129.1, 128.1, 123.3, 107.3, 90.0, 79.8, 71.1, 54.9, 50.6, 53.0, 49.3, 48.3, 41.1, 41.0, 40.8, 33.4, 28.6, 27.3, 26.1, 21.0, 20.4, 21.2, 19.1, 15.2; MS (API-ES) *m/z*: 414 $[M-C_5H_9O_2-C_{12}H_{19}O_2+H]^+$; de > 99%; mp: 132 °C;$[\alpha]_D^{20}$ +71 (*c* 0.5, DMF). Compound (2*R*,3*S*)-**1**: colorless solid. ^{1}H NMR (DMSO-d_6) 300 MHz δ 8.23 (s, 1H), 8.22 (s, 1H), 8.15 (s, 1H), 4.98 (s, 2H), 4.75 (d, J = 5.8 Hz, 1H), 2.96 (m, 2H), 2.78 (d, J = 11.5 Hz, 1H), 2.63, (ddd, J_1 = 12.3 Hz, J_2 = 8.9 Hz, J_3 = 3.6 Hz, 1H), 2.43 (dd, J_1 = 15.5 Hz, J_2 = 8.9 Hz, 1H), 2.33 (dd, J_1 = 11.8 Hz, J_2 = 2.3 Hz, 1H), 2.09 (br s, 1H), 1.88 (d, J = 11.5 Hz, 1H), 1.56 (d, J = 12.5 Hz, 1H), 1.33 (m, 1H), 1.19 (m, 1H); ^{13}C NMR (DMSO-d_6) 75 MHz δ: 203.5, 158.6, 149.7, 147.3, 132.4, 131.7, 128.3, 126.9, 121.8, 70.7, 60.1, 54.8, 45.6, 43.7, 34.2, 25.8; ee > 99%; mp: 170 °C (decomp.); $[\alpha]_D^{20}$ +9.4 (*c* 0.5, DMF); Anal. ($C_{16}H_{17}BrClN_3O_3$) C, H, N. Compound (2*S*,3*R*)-**1**: colorless solid.^{1}H NMR and ^{13}C NMR as for (2*R*,3*S*)-**1**; ee > 99%; mp: 160 °C (decomp.); $[\alpha]_D^{20}$ −10 (*c* 0.5, DMF); Anal. ($C_{16}H_{17}BrClN_3O_3$) C, H, N. Compound (2*R*,3*S*)-**1**, lactate: colorless solid.^{1}H NMR (DMSO-d_6) 300 MHz δ 8.25 (s, 1H), 8.23 (s, 1H), 8.17 (s, 1H), 5.00 (s, 2H), 3.94 (q, J = 6.8 Hz, 1H), 3.08 (ddd, J_1 = 9.9 Hz, J_2 = J_3 = 4.4 Hz, 1H), 3.02 (dd, J_1 = 15.9 Hz, J_2 = 4.0 Hz, 1H), 2.86 (d = J = 12.4 Hz, 1H), 2.76 (ddd, J_1 = 8.4 Hz, J_2 = J_3 = 3.8 Hz, 1H), 2.54 (dd, J_1 = 15.8 Hz, J_2 = 8.3 Hz, 1H), 2.45 (dd, J_1 = 11.7 Hz, J_2 = 2.6 Hz, 1H), 1.90 (m, 1H), 1.61 (m, 1H), 1.42–1.23 (m, 2H), 1.20 (d, J = 6.9 Hz, 3H); ^{13}C NMR (DMSO-d_6) 75 MHz δ 202.8, 176.8, 158.7, 149.7, 147.3, 132.4, 131.7, 128.3, 126.9, 121.8, 69.8, 66.1, 59.1, 54.7, 44.9, 42.6, 33.4, 24.5, 20.7; MS (API-ES) *m/z* 414 $[M+H]^+$; ee > 99%; mp: 161 °C (decomp.);$[\alpha]_D^{20}$ +11.8 (*c* 0.5, DMF). Compound (2*S*,3*R*)-**1**, lactate: colorless solid.[1]H NMR and ^{13}C NMR as for (2*R*,3*S*)-**1**, lactate; ee > 99%; mp: 163 °C (decomp.); $[\alpha]_D^{20}$ −11.0 (*c* 0.5, DMF) Determination of lactate content on a Metrohm 761 Compact Ion Chromatograph: Metrosep A Supp 5 (150 × 4.0 mm, 5 μm), $NaHCO_3$ (1 mM)/Na_2CO_3 (3.2 mM), flow 0.7 ml/min.
11. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC612821. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].
12. Najdrowski, M.; Heckeroth, A. R.; Wackwitz, C.; Gawlowska, S.; Mackenstedt, U.; Kliemt, D.; Daugschies, A. *Parasitol. Res.* **2007**, *101*, 161.