Bioorganic & Medicinal Chemistry 20 (2012) 2274–2281

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Impact of stereochemistry on the biological activity of novel oleandomycin derivatives

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ARTICLE INFO

Article history:
Received 5 December 2011
Revised 27 January 2012
Accepted 4 February 2012
Available online 13 February 2012

Keywords:
Macrolide
Configuration determination
Biological activity
Inflammation

ABSTRACT

A set of 8-methylene-, 8-methyl-, and 8-methyl-9-dihydro-oleandomycin derivatives having different combinations of stereochemistries at positions C-8 and/or C-9 have been prepared in a chemoselective and stereoselective manner and tested in vitro for antibacterial activity and inhibition of IL-6 production. Configurations of the stereocenters at C-8 and C-9 were determined using 2D NMR techniques. We have shown that change of stereochemistry at these positions can exert a major influence on antibacterial activity as well as IL-6 inhibition, providing novel macrolide derivatives with diminished antibacterial and potent anti-inflammatory activity. In addition, the anti-inflammatory activity observed in vitro was confirmed in an in vivo model of lipopolysaccharide-induced inflammation.

1. Introduction

The biological potential of macrolide antibiotics has not been fully explored yet, and new indications for this class of compounds have been recently discovered. Some natural and semisynthetic macrolides became particularly interesting because of their immunomodulatory activity.[1] There are examples of 14- and 15-membered macrolides where immunomodulatory and antibacterial activity can be separated by appropriate chemical derivatization.[2] Prompted by observation that some anti-bacterially active oleandomycin derivatives from our in-house macrolide collection showed potent in vitro inhibition of IL-6 cytokine production, we set as our goal to separate the anti-bacterial and immunomodulatory properties of oleandomycins by subtle changes of the macrolide aglycone.

Oleandomycin (**1**) is a natural 14-membered macrolide with an antibacterial spectrum similar to that of erythromycin A. It comprises two sugar moieties, α-L-oleandrose and β-D-desosamine, attached to positions C-3 and C-5 of the macrolactone ring, respectively. The distinguishing characteristic of oleandomycin is an exocyclic epoxide at position C-8. Chemical modifications of oleandomycin have been much less explored in comparison to the erythromycin family and can be classified in four categories: formation of oxime derivatives at C-9,[3] modifications of the exocyclic epoxide,[4] preparation of 9,10-anhydro derivatives,[5] and modifications of the 4″ position.[6]

Since macrolides are highly complex chiral molecules, inversion of stereochemistry at certain carbon atoms usually changes the macrolide properties, in particular their biological activity or PK/PD properties. It is known that the 8-(*R*)-methyl diastereomer **2** of oleandomycin is a more effective antibacterial agent than the 8-(*S*)-methyl isomer **3**, which can be regarded as antibacterially inactive.[4] We reasoned that the stereochemistry at positions C-8 and C-9 of 8-methylene-, 8-methyl-, and 8-methyl-9-dihydro-oleandomycins could have an influence on the potency of IL-6 cytokine inhibition. In order to prove this hypothesis selected derivatives of oleandomycin **2**–**9** (Scheme 1) having different absolute configurations at C-8 and/or C-9 were prepared. The syntheses have been achieved in a chemoselective and mostly stereoselective manner.

2. Results and discussion

2.1. Chemistry

The 8-epoxy group in **1** was first converted to an exocyclic double bond using chromium (II) chloride to afford the conjugated ketone **10**.[7] Chemoselective reduction of the 8-methylene group using Al/Hg[8] afforded only the 8-(*R*)-methyl isomer **2**[3] while Pd-catalyzed hydrogenation affords a diastereomeric mixture with a

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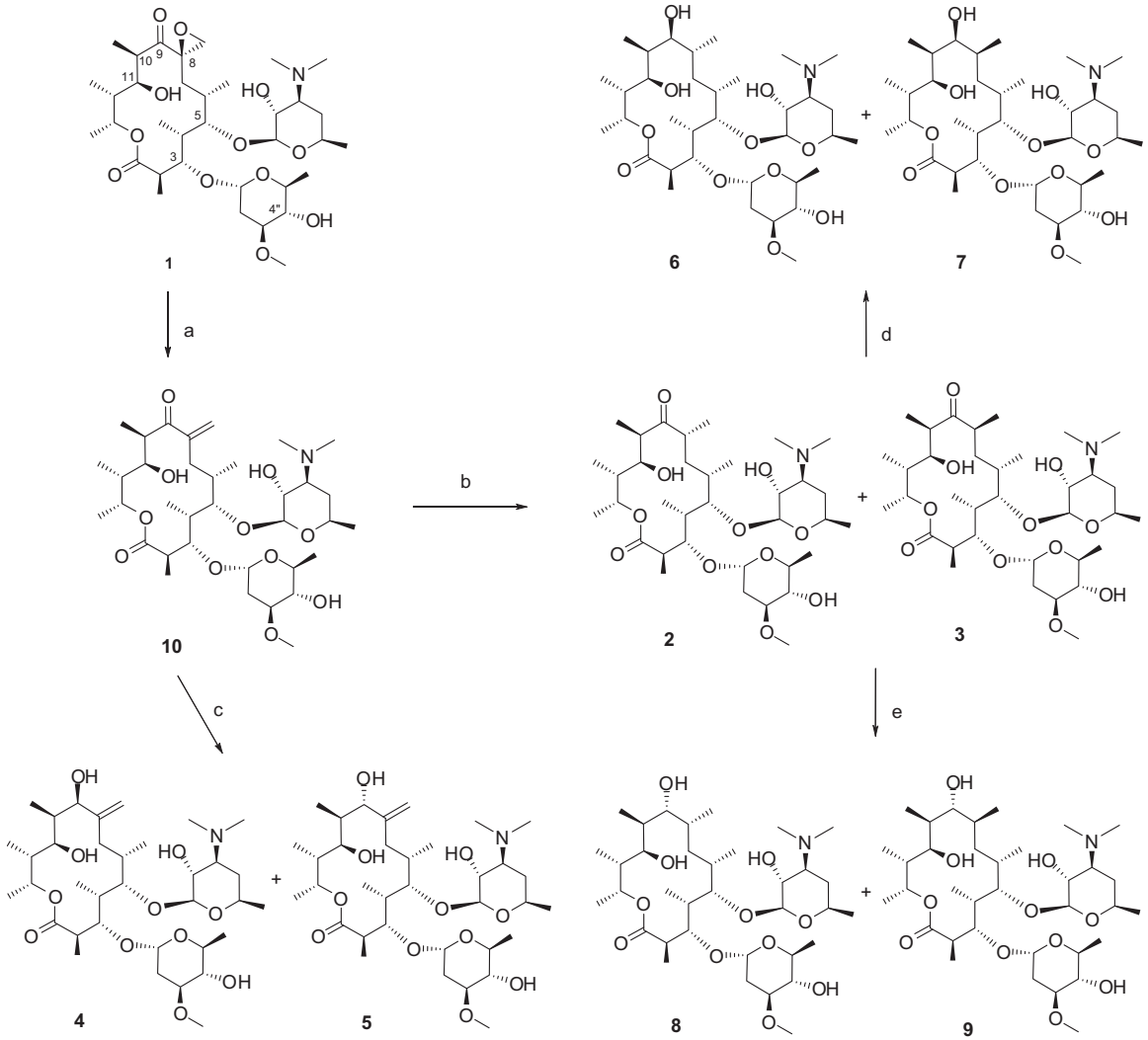
doi:10.1016/j.bmc.2012.02.013



Scheme 1. Reagents and conditions: (a) $CrCl_2$, acetone/water; (b) 1,4-enone reduction (major isomer **3**): H_2/Pd-C, ethanol, 6 bar (de 16%), or $Na_2S_2O_4$/$NaHCO_3$, dioxane/water (de up to 54%), or Ph_2SiH_2/$ZnCl_2$/$Pd(PPh_3)_4$, chloroform (de 40%); (c) 1,2-enone reduction (major isomer **4**): $NaBH_4$, methanol/water (de 94%) or $Na(OCH_3)_3BH$, methanol (de 94%); 1,2-enone reduction (major isomer **5**): $Na(OCOCH_3)BH$, glacial acetic acid (de 51%); (d) $NaBH_4$, methanol/water; e) $Na(OCOCH_3)_3BH$, tetrahydrofuran.

small excess of the (*S*)-isomer **3**[3] (de = 16%). In order to check the possibility of chemoselective and stereoselective 1,4-reduction of the conjugated ketone **10** to prepare the particularly antibacterially inactive 8-(*S*)-methyl isomer **3**, we have selected and examined six known methods for enone reductions. None of them allowed completely stereoselective reduction to either of the two 8-methyl isomers. Using $LiAlH_4$/CuI in THF,[9] $Co_2(CO)_8$ in monoglyme/water,[10] $[RhCl(cod)]_2$/Zn in dioxane/water,[11] and Me_2PhSiH/CuCl in 1,3-dimethylimidazolin-2-one[12] gave no desired 1,4-reduction reaction. This could be attributed to the high steric hindrance of complex macrolide scaffolds. Two methods worked well and gave a higher diastereomeric excess of the 8-(*S*)-methyl isomer than the usual Pd-catalyzed hydrogenation. While Ph_2SiH_2/$ZnCl_2$/$Pd(PPh_3)_4$ in chloroform[13] affords **3** with 40% de and 80% conversion, $Na_2S_2O_4$/$NaHCO_3$ in dioxane/water[14] affords **3** with an improved de (46% at 50 °C and 54% at 130 °C (MW)) along with very good conversion. Both isomers can be separated by column chromatography or used as a diastereomeric mixture in subsequent reactions. Chemoselective reduction of the C-9 ketone of 8-methylene oleandomycin **10** to 9-(*S*)-hydroxy-8-methylene oleandomycin **4**[15] was achieved with high diastereoselectivity using $NaBH_4$ or $Na(OCH_3)_3BH$. Both reagents attack the carbonyl group from the less hindered side of the macrolactone ring. On the other hand, the 9-(*R*)-hydroxy isomer **5** can be obtained in 50% de if $Na(OCOCH_3)_3BH$ is used. Such inversion of diastereoselectivity can be explained by prior coordination of the hydride anion by the C-11 hydroxyl (β-hydroxy) group, and thus direction of intramolecular reduction from the more hindered side.[16] Since this reagent is a mild reducing agent, a large excess of the reagent was needed to drive the reaction to completion.

If the 8-methylene group is already reduced to 8-methyl oleandomycin (compounds **2** and **3**), the reduction of C-9 ketone was achievable in a completely stereospecific manner using $NaBH_4$ and $Na(OCOCH_3)_3BH$. While $NaBH_4$ gave exclusively 9-(*S*)-hydroxy isomers **6**[17] and **7**, $Na(OCOCH_3)_3BH$ worked in an opposite manner, giving only the 9-(*R*)-hydroxy isomers **8** and **9** (Fig. 4). All 6 isomers (**4**–**9**) have been separated using column chromatography and their structures have been analysed in detail by 2D NMR. The NMR of oleandomycins **2** and **3** have been already described in detail.[3]

2.2. Structure confirmation

The NMR data (Tables 1 and 2) show that chemical shifts of the oleandomycins **4**–**9** vary mostly in the 'upper-left' region of the aglycone ring. One explanation for this is that the change of stereo-

Table 1
Comparison of selected ^{1}H NMR chemical shifts (δ/ppm, DMSO-d_6, 600 MHz, 80 °C,) of oleandomycins **4**–**9**

Proton No.	4	5	6	7	8	9
8-H	–	–	1.61	1.65	1.66	1.58
8=CH_2	5.20; 4.89	4.94; 4.73	–	–	–	–
8-CH_3	–	–	1.07	0.90	0.85	0.98
9-H	3.88	3.79	3.05	3.45	3.28	3.14
10-H	1.80	1.56	1.75	1.61	1.54	1.50
10-CH_3	0.93	0.87	0.91	0.92	0.82	0.85

Table 2
Selected proton-proton coupling constants (in Hz) for compounds **4**–**9**

$^3J_{n,m}$	4	5	6	7	8	9
2, 3	7.6	8.9	[a]	2.9	6.4	7.3
8, 9	–	–	[a]	2.5	2.6	7.9
9, 10	10.3	9.1	[a]	2.0	8.4	7.6

[a] Coupling constants unmeasurable due to signal broadening.

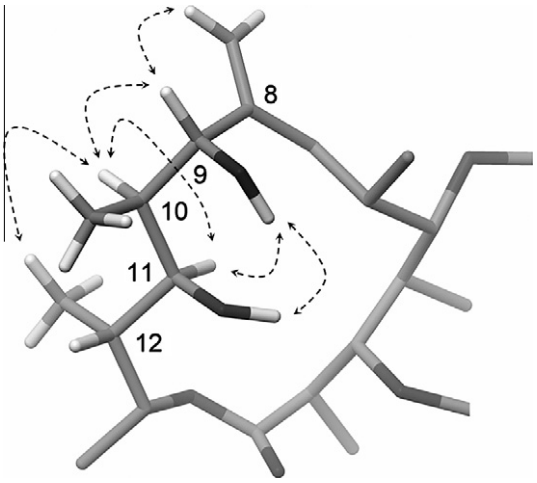


Figure 1. Diagram of the compound **4** macrolide ring showing strong nOe contacts. (Not energy-minimized, sugars and non-relevant hydrogen atoms deleted for clarity)

chemistry at positions C-8 and C-9 in compounds **6** – **9** influences both 2D and 3D structure of the macrolactone ring. All four isomers **6**, **7**, **8**, and **9**, in which both the ketone and double bond have been reduced, show broadened ^{1}H-NMR spectra in DMSO at 25 °C to a greater or lesser extent, very likely a result of increased flexibility of the macrolide ring upon reduction of the sp^2 carbons at C-8 and C-9. All configurational analyses were therefore performed on spectra recorded at 80 °C, in an effort to sharpen the spectra. The H-2/H-3 coupling constants (Table 2) indicate that isomer **7** has a classic folded-in[18] macrolide conformation, while **6**, **8** and **9** exhibit conformational averaging between folded-in and folded-out conformations at elevated temperature. The configurations at C-8, C-9 and C-10 could be inferred from nOe and conformational data as the macrolide ring closure constrains the allowed rotational states of this fragment and the absolute stereochemistry of C-10 was known and determined to be unchanged, as confirmed by nOe data, principally the strong 11-OH/10-Me enhancement present in all compounds.

To provide visual clarification of the NMR data, representative conformers, Figures 1-4 were generated manually by modifying X-ray crystallographic structures of suitable model compounds (CSD refcodes BBOLEA,[19] LIDBIJ[3] and ZATPAL[20]). Note however, that these structures are purely illustrative: they are not energy-minimized and represent only one possible conformer of what are conformationally flexible compounds.

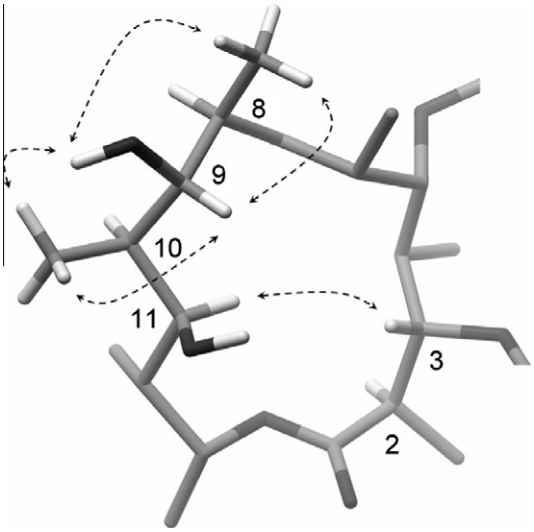


Figure 2. Diagram of the compound **9** macrolide ring showing strong nOe contacts. (Not energy-minimized, sugars and non-relevant hydrogen atoms deleted for clarity)

The stereochemistry of the previously reported compound **4**[15] and it's enantiomer **5**, were both confirmed by nOe experiments: compound **4** (Fig. 1) shows strong nOe's between H-9 and H-10, 10-Me, H-11 and H-14, whereas 9-OH shows strong nOe's to H-11 and 11-OH but does not H-10. This evidence is consistent with H-10 antiperiplanar to 9-OH. Compound **5**, in contrast, shows strong nOe's from H-9 to 10-Me and 11-OH but not to H-10, while 9-OH shows weak nOe's to 10-Me and H-10, consistent with H-9 antiperiplanar to H-10, confirming the stereochemistry as the 9-(*R*)-hydroxy isomer.

Isomer **9** (Fig. 2) exhibits large and equal coupling constants between H-9 and H-8, H-10, suggesting the same relative spatial arrangement of H-8, H-9 and H-10. Strong nOe's from both H-9 and 9-OH to 8-Me and 10-Me indicate both methyls bisect the H-9/C-9/9-OH angle, while the H-9/H-8 and H-9/H-10 nOe's are essentially non-existent. 9-OH gives only a weak nOe to H-10. This evidence is consistent with both H-8and H-10 being antiperiplanar to H-9 with 8-Me and 10-Me adopting pseudo-equatorial positions. Unfortunately, the expected H-8/H-10 nOe could not been seen as the close chemical shifts of these protons resulted in any possible cross-peak being lost in the NOESY spectrum diagonal.

In contrast to isomer **9**, isomer **7** (Fig. 3) shows equal but small couplings between H-9 and H-8, H-10. Strong nOe's were observed from H-9 to both 8-Me and 10-Me and from 9-OH to these two methyls, while H-9 also shows strong nOe's to H-8 and H-10. This evidence is consistent with the methyls adopting pseudo-equatorial positions, both synclinal to H-9 and 9-OH, H-9 synclinal to H-8 and H-10, with 9-OH adopting a pseudo-axial orientation. No nOe's were observed from 9-OH to H-8 or H-10, as expected. An nOe between H-9 and H-6 is consistent with the pseudo-axial position of H-9. Potential nOe's between H-8 and H-10 were obscured by the NOESY diagonal.

Isomer **6** (Fig. 4) showed selective signal broadening in both the ^{1}H NMR and ^{13}C NMR spectra, even at elevated temperature, localized from positions 3 to 9 on the macrolide ring, along with some signals of the desosamine ring. It has previously been established that the desosamine ring has limited conformational mobility about the glycosidic linkage in erythromycins.[18] However, in the case of isomer **6**, the additional conformational flexibility (induced by reduction of C-9 in combination with the stereochemistry of

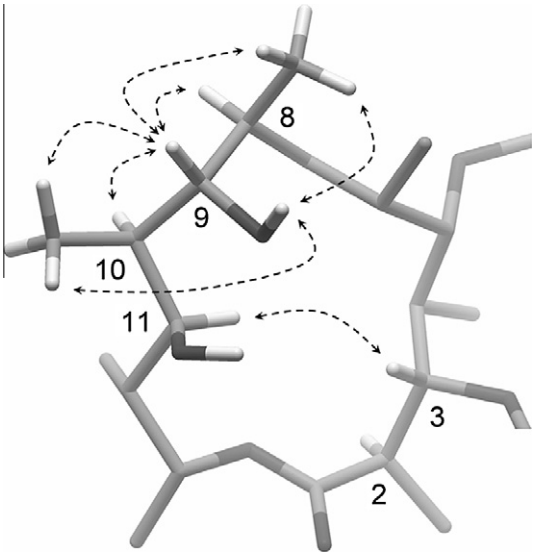


Figure 3. Diagram of the compound **7** macrolide ring showing strong nOe contacts. (Not energy-minimized, sugars and non-relevant hydrogen atoms deleted for clarity)

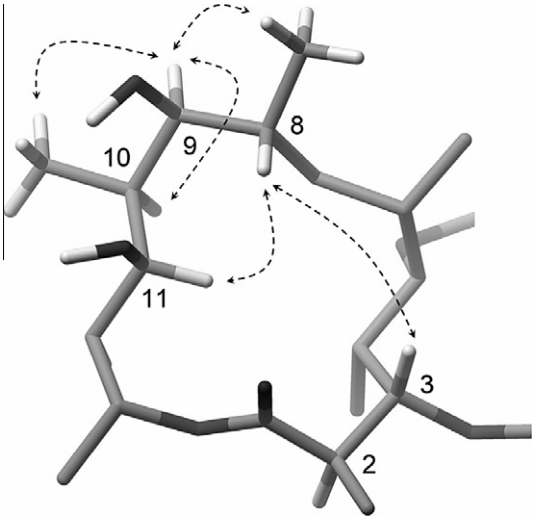


Figure 4. Diagram of the compound **6** macrolide ring showing strong nOe contacts. (Not energy-minimized, sugars and non-relevant hydrogen atoms deleted for clarity)

C-8/C-9) may allow the desosamine to sample a larger conformational space, albeit with slow dynamics. Unfortunately, the signal broadening prevented extraction of the H-8/H-9 and H-9/H-10 couplings. Strong nOe's were observed from H-9 to both H-10 and 10-Me, suggesting H-9 is synclinal to both, with 9-OH in a pseudo-axial position. A strong nOe was seen between H-9 and 8-Me, but none between H-10/8-Me or H-8/10-Me. This would be consistent with 8-Me antiperiplanar to 9-OH with H-8/H-9 synclinal rather than H-8/9-OH antiperiplanar and H-9/8-Me synclinal. H-8 showed strong nOe's to H-3 and H-11, consistent with H-8 being endo to the macrolide ring.

Isomer **8** shows a large H-9/H-10 coupling constant, similar to that seen in isomer **9**, but a small coupling constant between H-9 and H-8, indicative of inversion of stereochemistry at C-8. Strong nOe's between H-9 and 10-Me, medium between 9-OH and H-10 and weak between H-9 and H-10 are all consistent with H-9/H-10 antiperiplanar and the H-9/10-Me, 9-OH/H-10 pairs being synclinal. A strong nOe was observed between H-9 and H-8, ruling out an antiperiplanar arrangement, a conclusion supported by a medium nOe between H-10 and 8-Me. However, a moderate nOe observed between H-9 and 8-Me could be explained by a fast local conformational equilibrium in which 8-Me moves from antiperiplanar to anticlinal (or even synclinal) to H-9. In addition, a medium nOe between H-9 and H-6 is not consistent with a single conformer which could simultaneously fit all the previously-described evidence, strongly suggesting a rapid conformational equilibrium in which the C-6 to C-10 region (or larger) could undergo an endo-/exo-folding motion.

Table 3
Biological activities of oleandomycins **2–9**

	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**
MIC *(μg/ml)*								
S.aureus	4	>64	>64	>64	>64	>64	>64	>64
S.pneumoniae	0.25	>64	>64	>64	4	>64	>64	>64
S.pyogenes	0.5	>64	>64	>64	>64	>64	>64	>64
M.catarrhalis	2	>64	>64	>64	>64	>64	>64	>64
H. influenzae	>64	>64	>64	>64	>64	>64	>64	>64
IL-6 inhibition (50 μM)	**69%**	42%	13%	13%	**61%**	12%	2%	2%

2.3. Biological activity

The antimicrobial activity of all eight oleandomycin analogues was assessed on a panel of five common respiratory pathogens (*Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Moraxella catarrhalis* and *Haemophilus influenzae*). Inhibition of LPS-stimulated IL-6 cytokine production in vitro was measured for all compounds as well. As expected, the stereochemistry at positions 8 and 9 had a large impact on both activities (Table 3). Regarding 9-keto analogues, the 8-(*R*)-methyl isomer **2** had the strongest antibacterial (lowest MIC values) and anti-inflammatory activity (highest IL-6 inhibition). The 9-(*R*)- and 9-(*S*)-dihydro-8-methylene compounds **4** and **5** had neither antibacterial nor anti-inflammatory activity. The most interesting compounds were 9-dihydro-8-methyl distereoisomers **6–9** among which only one combination (9-(*S*)-hydroxy-8-(*R*)-methyl diastereoisomer **6**) significantly inhibited LPS-stimulated IL-6 production. Interestingly, compound **6** is the only one that retained some weak residual antibacterial activity on *S. pneumoniae*. Inhibition of cytokine production was not caused by reduction in cell viability as compounds had no cytotoxic effects on THP-1 cells even at the highest concentration tested, 100 μM.

Since IL-6 is a pleiotropic cytokine influencing a wide range of biological activities including inflammatory reactions, possible anti-inflammatory potential of the novel oleandomycin compounds was assessed in the LPS-induced pulmonary neutrophilia model in vivo. As expected, differences in anti-inflammatory activities observed in vitro were confirmed for a pair of diastereoisomers (compounds **6** and **9**) in vivo (Fig. 5). Compound **6** strongly decreased neutrophil numbers in bronchoalveolar lavage fluid following LPS challenge while its diastereoisomer (compound **9**) was only weakly active.

3. Conclusion

In conclusion, a set of 8-methylene-, 8-methyl-, and 8-methyl-9-dihydro-oleandomycins differed in stereochemistries at positions C-8 and/or C-9 were prepared in a chemoselective and stereoselective manner. Configurations of stereocenters at C-8 and C-9 were determined using 2D NMR techniques. In vitro testing of all compounds for antibacterial activity and IL-6 inhibition shown that even a small structural difference such as different

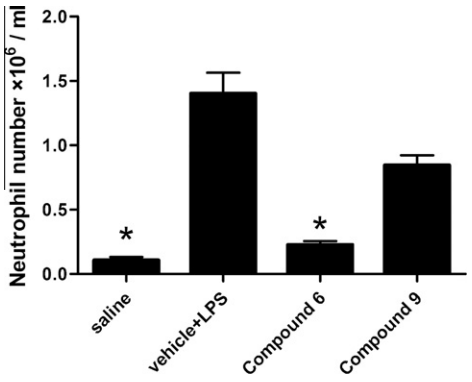


Figure 5. Neutrophil number in BALF 24 h after LPS challenge. Compounds were administered intraperitoneally 2 h prior LPS challenge at a dose of 100 mg/kg in mice. Data are presented as means ± SEM. Asterisk represents p <0.05, Kruskal-Wallis test followed by Dunn's multiple comparison test.

stereochemistry at certain positions in these complex molecules can exert a major influence on their biological effects. Moreover, one novel oleandomycin derivative with diminished antibacterial and potent anti-inflammatory activity was identified. In addition, the anti-inflammatory activity observed in vitro was confirmed in an in vivo model of LPS-induced inflammation.

4. Experimental section

All solvents and reagents were used as supplied, unless noted otherwise. HRMS (ESI) were recorded on Micromass Qtof2. One and two-dimensional NMR spectra (1H, APT, COSY, NOESY, ROESY, edited HSQC and HMBC) were recorded on Bruker Avance III 600 or Avance I 400 spectrometers, both equipped with 5 mm probes with z-gradient accessory. All spectra were recorded using standard Bruker pulse sequences, on compounds dissolved in DMSO-d_6 at the indicated temperature with TMS as the internal standard.

4.1. 1,4-Reduction of ketone 10

H_2/Pd-C: Ketone **10**[7] (2.55 g, 3.8 mmol) was dissolved in EtOH (36 ml) and 10% Pd/C (1.50 g) was added. Hydrogen was applied at 6 bar, at room temperature and with shaking for 20 h. The catalyst was filtered off (Büchner funnel, blue spot) and the solvent evaporated affording a diastereomeric mixture of **2** and **3** (2.40 g, de of **3** = 16%) used as is in a subsequent reactions. Pure isomers **2** and **3** for biological testing were obtained by silica-gel column chromatography.[3]

$Na_2S_2O_4$/$NaHCO_3$: Ketone **10** (50 mg, 0.075 mmol) was dissolved in 1,4-dioxane (0.5 ml) and $NaHCO_3$ (20.3 mg; 0.24 mmol) was dissolved in water (0.5 ml). The two solutions were mixed and $Na_2S_2O_4$ (9.3 mg; 0.05 mmol) was added. Within 1 h, 3 more portions of $Na_2S_2O_4$ (9.3 mg; 0.05 mmol) were added. Reaction was terminated by adding some water. The solution was extracted with CH_2Cl_2. Extracts were dried on anhydrous Na_2SO_4 for 5 min, filtered, and evaporated.

(a) temperature: 50 °C, reaction time: 5 h; de of **3** = 46%.
(b) temperature: 0 °C, reaction time: 2 h; only starting ketone **10**.
(c) room temperature, reaction time: overnight; equimolar diastereomeric mixture of **2** and **3**; 8% of starting ketone **10**.
(d) MW assisted: 130 °C, 1 min and 11 sec (absorption level – high; maximum pressure: 2 bar); de of **3** = 54%.

Ph_2SiH_2/$ZnCl_2$/$Pd(PPh_3)_4$: Ketone **10** (50 mg, 0.075 mmol), $ZnCl_2$ (20 mg, 0.15 mmol), Ph_2SiH_2 (84 μl, 0.45 mmol) and $[Pd(PPh_3)_4]$ (15 mg) were dissolved and stirred in dry CH_2Cl_2 (0.5 ml) at room temperature under N_2. Reaction was terminated after 5 h by adding some water and CH_2Cl_2. The layers were separated at pH ≈1.8. The organic layer was extracted with CH_2Cl_2 at pH ≈13.0. Organic extracts were dried on anhydrous Na_2SO_4 for 5 min. Salt was filtered off and the solvent evaporated, affording 15 mg of a diastereomeric mixture of **2** and **3** (de of **3** = 40%) along with 3.6% (according to LC–MS) of 1,2 + 1,4 reduction side product.

4.2. 1,2-Reduction of ketone 10

$NaBH_4$: To a solution of ketone **10** (1.0 g, 1.5 mmol) in MeOH (40 ml)/H_2O (40 ml) $NaBH_4$ (168 mg, 4.5 mmol) was added in one portion. After 3 h the reaction was terminated by the addition of some water and CH_2Cl_2, the pH being adjusted to 2.3 using hydrochloric acid. After 5 min, the pH was adjusted to 13.0 using sodium hydroxide and the layers separated. The organic layer was dried on anhydrous Na_2SO_4 for 5 min. Salt was filtered off and the solvent evaporated, affording 1.06 g of a diastereomeric mixture of **4** and **5** (de of **4** = 94%). The product was purified by column chromatography (silica-gel as the stationary phase and CH_2Cl_2/MeOH/NH_4OH (90:9:1.5) as eluent. A total of 146 mg (15%) of the compound **4** was obtained as a white foam. ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C): δ = 0.77 (d, J = 7.2 Hz, 3H, 12Me), 0.93 (d, J = 7.0 Hz, 3H, 10Me), 1.08 (d, J = 7.0 Hz, 3H, 2Me), 1.13 (m, 1H, 4′ax), 1.10 (d, J = 7.2 Hz, 3H, 4Me), 1.12 (d, J = 7.2 Hz, 3H, 6Me), 1.13 (d, J = 7.2 Hz, 3H, 5′Me), 1.15 (d, J = 6.6 Hz, 3H, 14), 1.21 (d, J = 6.3 Hz, 3H, 5″Me), 1.43 (m, 1H, 2″ax), 1.44 (m, 1H, 12), 1.51 (dd, J = 18.0, 9.8 Hz, 1H, 7b), 1.60 (dq, J = 9.6, 7.2 Hz, 1H, 4), 1.63 (ddd, J = 12.7, 4.1, 2.0 Hz, 1H, 4′eq), 1.80 (m, J = 6.9, 6.9, 6.9, 4.2, 1.2 Hz, 1H, 10), 1.94 (d, J = 17.8 Hz, 1H, 7a), 2.09 (dq, J = 8.9, 6.8 Hz, 1H, 6), 2.20 (ddd, J = 12.9, 4.7, 1.6 Hz, 1H, 2″eq), 2.24 (s, 6 H, 3′NMe), 2.47 (ddd, J = 12.4, 10.1, 4.2 Hz, 1H, 3′ax), 2.64 (quin, J = 7.2 Hz, 1H, 2), 2.94 (td, J = 8.9, 5.6 Hz, 1H, 4″ax), 3.08 (ddd, J = 9.6, 8.2, 1.4 Hz, 1H, 2′ax), 3.32 (ddd, J = 11.5, 8.7, 4.7 Hz, 1H, 3″eq), 3.34 (s, 3H, 3″OMe), 3.41 (ddd, J = 8.9, 2.6, 1.2 Hz, 1H, 11), 3.43 (d, J = 10.3 Hz, 1H, 5), 3.46 (m, J = 10.8, 6.3, 6.3, 6.3, 1.9 Hz, 1H, 5′ax), 3.51 (d, J = 3.0 Hz, 1H, 11OH), 3.60 (dq, J = 9.2, 6.1 Hz, 1H, 5″ax), 3.63 (d, J = 7.9 Hz, 1H, 3), 3.88 (dd, J = 10.3, 4.7 Hz, 1H, 9), 4.15 (d, J = 7.3 Hz, 1H, 1′ax), 4.63 (d, J = 5.9 Hz, 1H, 9OH), 4.76 (m, 1H, 4″OH), 4.88 (dd, J = 4.2, 1.2 Hz, 1H, 1″eq), 4.89 (br s, 1H, 8CH_2b), 5.20 (s, 1H, 8CH_2a), 5.27 (q, J = 6.6 Hz, 1H, 13) ppm; ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C): δ = 8.0 (12Me), 8.9 (10Me), 9.0 (4Me), 13.8 (2Me), 17.4 (5″Me), 18.1 (6Me), 18.4 (14), 20.5 (5′Me), 29.9 (4′), 31.1 (6), 31.5 (7), 34.2 (2″), 34.6 (10), 39.9 (3′NMe), 42.2 (4), 42.6 (12), 44.1 (2), 56.0 (3″OMe), 64.5 (3′), 67.8 (5′), 68.6 (5″), 69.9 (13), 70.2 (2′), 70.3 (11), 75.4 (4″), 77.1 (9), 77.3 (3″), 79.8 (3), 83.2 (5), 98.6 (1″), 104.0 (1′), 107.3 (8CH_2), 148.9 (8), 174.3 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{64}NO_{11}$: 674.4479, found: 674.4500.

$Na(OCH_3)_3BH$: To a solution of ketone **10** (50 mg, 0.075 mmol) in MeOH (2.5 ml) $Na(OCH_3)_3BH$ (28.4 mg, 0.24 mmol) was added in one portion. After 3.5 h the reaction was terminated by the addition of some water and CH_2Cl_2, the pH being set to 2.3 (using hydrochloric acid). After 5 min, the pH was set to 12.5 using sodium hydroxide and the layers separated. The organic layer was dried on anhydrous Na_2SO_4 for 5 min, filtered, and solvent evaporated, affording 44 mg of crude product as a mixture of **4** (75%), **5** (2.1%), starting ketone **10** (17.2%), and 1,2+1,4-reduction product (5.7%) (according to LC–MS).

$Na(OCOCH_3)_3BH$: To a solution of ketone **10** (1.0 g, 1.5 mmol) in glacial HOAc (50 ml), $Na(OCOCH_3)_3BH$ (18.7 g, 90 mmol) was added in 12 portions, one 5-equiv portion every 30 min. After 6.5 h the reaction was terminated by the addition of some water and CH_2Cl_2, the pH being set to 2.1 (using hydrochloric acid). After 5 min, the pH was set to 10.0 (using sodium hydroxide) and the

layers separated. The organic layer was dried on anhydrous Na_2SO_4 for 5 min, filtered, and the solvent evaporated, affording 920 mg of a diastereomeric mixture of **4** and **5** (de of **5** = 51%). The product **5** was separated by column chromatography (silica-gel, flash) with CH_2Cl_2/MeOH/NH_4OH (90:9:1.5) as an eluent to afford 611 mg (61%) of **5** as a white foam. ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C): δ = 0.75 (d, J = 7.2 Hz, 3H, 12Me), 0.87 (d, J = 6.6 Hz, 3H, 10Me), 1.09 (d, J = 7.2 Hz, 3H, 2Me), 1.10 (d, J = 7.0 Hz, 3H, 4Me), 1.15 (m, 1H, 4′ax), 1.14 (d, J = 7.1 Hz, 3H, 6Me), 1.14 (d, J = 5.9 Hz, 3H, 5′Me), 1.17 (d, J = 6.6 Hz, 3H, 14), 1.21 (d, J = 6.1 Hz, 3H, 5″Me), 1.43 (m, 1H, 12), 1.45 (m, 1H, 2″ax), 1.55 (m, 1H, 4), 1.56 (m, 1H, 10), 1.66 (ddd, J = 12.6, 4.1, 1.9 Hz, 1H, 4′eq), 1.84 (m, 2H, 7b, 7a), 1.88 (m, 1H, 6), 2.19 (ddd, J = 13.0, 4.7, 1.6 Hz, 1H, 2″eq), 2.28 (s, 6 H, 3′NMe), 2.53 (ddd, J = 12.4, 9.9, 3.8 Hz, 1H, 3′ax), 2.67 (dq, J = 8.9, 7.0 Hz, 1H, 2), 2.95 (qd, J = 8.9, 5.8 Hz, 1H, 4″ax), 2.97 (d, J = 9.8 Hz, 1H, 11), 3.02 (d, J = 5.9 Hz, 1H, 11OH), 3.12 (dd, J = 10.1, 7.3 Hz, 1H, 2′ax), 3.31 (ddd, J = 11.3, 8.5, 4.7 Hz, 1H, 3″eq), 3.34 (s, 3H, 3″OMe), 3.40 (d, J = 9.9 Hz, 1H, 5), 3.47 (m, J = 10.8, 6.1, 6.1, 6.1, 1.9 Hz, 1H, 5′ax), 3.55 (d, J = 8.9 Hz, 1H, 3), 3.61 (dq, J = 9.2, 6.1 Hz, 1H, 5″ax), 3.79 (dd, J = 9.1, 4.4 Hz, 1H, 9), 4.05 (br s, 1H, 2′OH), 4.16 (d, J = 7.2 Hz, 1H, 1′ax), 4.29 (d, J = 3.8 Hz, 1H, 9OH), 4.73 (q, J = 1.6 Hz, 1H, 8CH_2b), 4.77 (d, J = 5.2 Hz, 1H, 4″OH), 4.87 (dd, J = 3.8, 1.3 Hz, 1H, 1″eq), 4.94 (q, J = 1.0 Hz, 1H, 8CH_2a), 5.34 (q, J = 6.5 Hz, 1H, 13) ppm; ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C): δ = 8.2 (10Me), 8.4 (12Me), 8.9 (4Me), 14.2 (2Me), 17.4 (5″Me), 18.4 (14), 18.5 (6Me), 20.5 (5′Me), 28.4 (7), 29.9 (4′), 30.9 (6), 34.2 (2″), 37.4 (10), 39.8 (3′NMe), 42.0 (4), 42.4 (12), 44.2 (2), 56.1 (3″OMe), 64.5 (3′), 67.7 (5′), 68.7 (5″), 69.8 (13), 69.9 (11), 70.1 (2′), 75.4 (4″), 77.1 (3″), 79.8 (9), 80.4 (3), 83.3 (5), 98.8 (1″), 103.9 (1'), 109.3 (8CH_2), 149.5 (8), 174.6 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{64}NO_{11}$: 674.4479, found: 674.4460.

4.3. Reduction of a diastereomeric mixture of 2 and 3

$NaBH_4$: To a solution of a diastereomeric mixture of **2** and **3** (1.07 g, 1.6 mmol) in MeOH (40 ml)/H_2O (40 ml) $NaBH_4$ (180 mg; 4.8 mmol) was added in one portion. Reaction was terminated after 2 h. MeOH was evaporated and some H_2O and CH_2Cl_2 was added. pH was adjusted to pH ≈ 2 and after a few minutes it was raised to pH ≈10. Layers were separated and organic extracts dried on anhydrous Na_2SO_4, filtered, and solvent evaporated affording a diastereomeric mixture of **6** and **7** (950 mg). The mixture was separated by column chromatography (silica-gel, flash) with CH_2Cl_2/MeOH/NH_4OH (90:9:1.5) as an eluent to afford homogeneous fractions of compounds **6** (269 mg) and **7** (218 mg) as a white foams. Compound **6**: ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C): δ = 0.71 (ddd, J = 14.0, 11.9, 5.6 Hz, 1H, 7b), 0.79 (d, J = 7.0 Hz, 3H, 12Me), 0.91 (d, J = 7.0 Hz, 3H, 10Me), 0.95 (d, J = 5.9 Hz, 3H, 6Me), 1.00 (d, J = 7.2 Hz, 3H, 4Me), 1.07 (d, J = 7.0 Hz, 3H, 2Me), 1.07 (d, J = 6.3 Hz, 3H, 8Me), 1.17 (d, J = 6.1 Hz, 3H, 5′Me), 1.19 (d, J = 6.6 Hz, 3H, 5″Me), 1.20 (d, J = 6.1 Hz, 3H, 14), 1.24 (m, 1H, 7a), 1.44 (m, 1H, 4′ax), 1.46 (m, 1H, 12), 1.48 (ddd, J = 13.3, 10.8, 4.0 Hz, 1H, 2″ax), 1.61 (m, 1H, 8), 1.73 (m, 1H, 4), 1.75 (m, 1H, 10), 1.94 (m, 1H, 6), 1.96 (dd, J = 11.9, 2.6 Hz, 1H, 4′eq), 2.14 (ddd, J = 13.1, 4.7, 2.1 Hz, 1H, 2″eq), 2.68 (m, 1H, 2), 2.70 (s, 6 H, 3′NMe), 2.99 (t, J = 8.7 Hz, 1H, 4″ax), 3.05 (br s, 1H, 9), 3.19 (m, 1H, 3′ax), 3.36 (m, 1H, 2′ax), 3.36 (s, 3H, 3″OMe), 3.44 (ddd, J = 11.5, 8.2, 4.7 Hz, 1H, 3″eq), 3.60 (d, J = 9.8 Hz, 1H, 11), 3.62 (dq, J = 8.9, 6.1 Hz, 1H, 5″ax), 3.73 (br s, 1H, 3), 3.77 (m, 1H, 5′ax), 4.06 (br s, 1H, 11OH), 4.41 (d, J = 6.8 Hz, 1H, 1″eq), 4.81 (br s, 1H, 9OH), 4.91 (br s, 1H, 4″OH), 4.92 (dd, J = 3.8, 2.1 Hz, 1H, 1′ax), 5.21 (q, J = 6.3 Hz, 1H, 13), 5.34 (m, 1H, 2′OH) ppm; ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C): δ = 7.8 (12Me), 8.8 (4Me), 9.5 (10Me), 11.6 (2Me), 16.6 (8Me), 17.5 (5″Me, 14), 20.2 (5′Me), 30.4 (4′), 32.1 (10), 33.5 (2″), 33.7 (7), 35.2 (8), 35.9 (6), 39.2 (3′NMe), 40.3 (4), 41.7 (12), 42.7 (2), 56.1 (3″OMe), 65.0 (3′), 66.6 (5′), 68.6 (2′), 68.8 (), 68.9 (5″), 69.4 (13), 69.8 (11), 75.0 (4″), 77.0 (3″), 77.4 (3), 80.8 (9), 95.1 (1″), 102.3 (1′), 175.0 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{66}NO_{11}$: 676.4636, found: 676.4637.

Compound **7**: ^{1}H NMR (400 MHz, DMSO-d_6, 80 °C): δ = 0.79 (d, J = 7.1 Hz, 3H, 12Me), 0.88 (d, J = 7.1 Hz, 3H, 6Me), 0.90 (d, J = 7.3 Hz, 3H, 8Me), 0.92 (d, J = 7.3 Hz, 3H, 10Me), 1.05 (d, J = 6.9 Hz, 3H, 4Me), 1.08 (d, J = 7.1 Hz, 3H, 2Me), 1.15 (m, 1H, 4′ax), 1.12 (d, J = 6.1 Hz, 3H, 5′Me), 1.18 (d, J = 6.2 Hz, 6 H, 5″Me, 14), 1.36 (ddd, J = 12.7, 11.8, 3.7 Hz, 1H, 2″ax), 1.35 - 1.43 (m, 1H, 7b), 1.47 (br s, 1H, 12), 1.48 (m, 1H, 7a), 1.61 (m, 1H, 10), 1.63 (m, 1H, 4′eq), 1.65 (m, 1H, 8), 1.86 (quin, J = 7.6 Hz, 1H, 4), 2.14 (m, 1H, 6), 2.15 (ddd, J = 12.9, 4.9, 1.4 Hz, 1H, 2″eq), 2.24 (s, 6 H, 3′NMe), 2.45 (ddd, J = 12.0, 9.9, 4.0 Hz, 1H, 3′ax), 2.57 (qd, J = 6.9, 2.9 Hz, 1H, 2), 2.93 (td, J = 8.9, 5.3 Hz, 1H, 4″ax), 3.11 (ddd, J = 9.6, 7.3, 2.3 Hz, 1H, 2′ax), 3.35 (m, 1H, 3″eq), 3.34 (s, 3H, 3″OMe), 3.42 (dd, J = 8.7, 1.3 Hz, 1H, 5), 3.45 (ddd, J = 5.3, 2.5, 2.0 Hz, 1H, 9), 3.51 (m, J = 10.8, 5.9, 5.9, 5.9, 1.8 Hz, 1H, 5′ax), 3.65 (dq, J = 9.1, 6.3 Hz, 1H, 5″ax), 3.77 (br s, 1H, 2′OH), 3.89 (br s, 1H, 3), 3.95 (d, J = 9.5 Hz, 1H, 11), 3.99 (d, J = 2.2 Hz, 1H, 11OH), 4.17 (d, J = 7.3 Hz, 1H, 1′ax), 4.67 (d, J = 5.1 Hz, 1H, 4″OH), 4.72 (d, J = 5.4 Hz, 1H, 9OH), 4.88 (dd, J = 3.7, 1.2 Hz, 1H, 1″eq), 5.14 (qd, J = 6.6, 1.3 Hz, 1H, 13) ppm; ^{13}C NMR (101 MHz, DMSO-d_6, 80 °C): δ = 8.3 (12Me), 10.0 (4Me), 11.0 (2Me), 11.3 (10Me), 13.0 (6Me), 17.0 (8Me), 17.2 (5″Me), 17.3 (14), 20.6 (5′Me), 30.1 (4′), 33.5 (7), 34.1 (2″), 34.9 (6), 35.4 (8), 37.0 (10), 39.9 (3′NMe), 40.8 (12), 41.8 (4), 44.1 (2), 55.9 (3″OMe), 64.5 (3′), 67.8 (5′), 68.4 (5″), 69.8 (13), 70.0 (2′), 70.5 (11), 75.4 (4″), 76.8 (3), 77.4 (3″), 80.1 (9), 83.5 (5), 96.8 (1″), 104.7 (1′), 174.9 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{66}NO_{11}$: 676.4636, found: 676.4631.

$Na(OCOCH_3)_3BH$: To a solution of a diastereomeric mixture of **2** and **3** (1.19 g, 1.8 mmol) in dry THF acetic acid (4.12 ml, 60 mmol, in 4 portions) and $Na(OCOCH_3)_3BH$ (11.4 g, 54 mmol, in 3 portions) were added every 30 min. Reaction mixture was allowed to stir overnight. Reaction was terminated by the addition of some H_2O and CH_2Cl_2. pH was adjusted to pH ≈ 2.3 and after a few minutes it was raised to pH ≈11.5. Layers were separated and organic extracts dried on anhydrous Na_2SO_4, filtered, and solvent evaporated affording a diastereomeric mixture of **8** and **9** (1.25 g). A total of 255 mg of the crude product was purified by preparative HPLC-MS to give **8** (58 mg) and **9** (146 mg) as a white foams.

Compound **8**: ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C): δ = 0.79 (d, J = 7.2 Hz, 3H, 12Me), 0.82 (d, J = 6.8 Hz, 3H, 10Me), 0.85 (d, J = 7.3 Hz, 3H, 8Me), 0.95 (d, J = 6.8 Hz, 3H, 6Me), 1.12 (m, 1H, 4′ax), 1.08 (d, J = 6.8 Hz, 3H, 4Me), 1.09 (d, J = 7.0 Hz, 3H, 2Me), 1.13 (d, J = 6.1 Hz, 3H, 5′Me), 1.18 (d, J = 6.4 Hz, 3H, 5″Me), 1.19 (d, J = 6.8 Hz, 3H, 14), 1.23 (ddd, J = 13.4, 8.4, 6.6 Hz, 1H, 7b), 1.29 (ddd, J = 13.4, 9.1, 4.2 Hz, 1H, 7a), 1.41 (ddd, J = 12.6, 11.7, 3.8 Hz, 1H, 2″ax), 1.52 (quin, J = 7.5 Hz, 1H, 12), 1.54 (quin, J = 7.2 Hz, 1H, 10), 1.63 (ddd, J = 12.7, 3.8, 1.6 Hz, 1H, 4′eq), 1.66 (m, 1H, 8), 1.76 (dq, J = 9.6, 7.2 Hz, 1H, 4), 181 (m, 1H, 6), 2.19 (ddd, J = 12.6, 4.9, 1.2 Hz, 1H, 2″eq), 2.24 (s, 6 H, 3′NMe), 2.46 (ddd, J = 12.0, 10.3, 4.2 Hz, 1H, 3'ax), 2.66 (quin, J = 6.8 Hz, 1H, 2), 2.92 (t, J = 8.9 Hz, 1H, 4″ax), 3.10 (dd, J = 9.9, 7.3 Hz, 1H, 2′ax), 3.28 (dd, J = 8.4, 2.6 Hz, 1H, 9), 3.32 (ddd, J = 11.7, 8.5, 4.7 Hz, 1H, 3″eq), 3.34 (s, 3H, 3″OMe), 3.38 (d, J = 9.2 Hz, 1H, 11), 3.38 (d, J = 10.4 Hz, 1H, 5), 3.45 (m, J = 11.0, 6.1, 6.1, 6.1, 1.6 Hz, 1H, 5′ax), 3.52 (br s, 1H, 11OH), 3.59 (dq, J = 9.1, 6.1 Hz, 1H, 5″ax), 3.78 (br s, 1H, 9OH), 3.76 (d, J = 5.9 Hz, 1H, 3), 3.86 (br s, 1H, 2′OH), 4.15 (d, J = 7.2 Hz, 1H, 1′ax), 4.76 (br s, 1H, 4″OH), 4.88 (d, J = 3.1 Hz, 1H, 1″eq), 5.30 (q, J = 6.5 Hz, 1H, 13) ppm; ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C): δ = 8.8 (12Me), 9.0 (10Me), 9.6 (4Me), 13.1 (2Me), 15.2 (8Me), 17.3 (5″Me), 17.5 (6Me), 18.0 (14), 20.6 (5′Me), 29.9 (4′), 30.4 (7), 31.3 (6), 34.1 (2″), 36.2 (8), 37.3 (10), 39.9 (3′NMe), 42.4 (12), 42.8 (4), 44.3 (2), 56.0 (3″OMe), 64.6 (3′), 67.8 (5′), 68.6 (5″), 70.1

(2′, 11), 70.7 (13), 75.4 (4″), 75.7 (9), 77.2 (3″), 78.4 (3), 83.6 (5), 98.0 (1″), 104.1 (1′), 175.5 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{66}NO_{11}$: 676.4636, found: 676.4628.

Compound **9**: ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C): δ = 0.76 (d, J = 7.0 Hz, 3H, 12Me), 0.85 (d, J = 6.6 Hz, 3H, 10Me), 0.91 (m, 1H, 7b), 0.98 (d, J = 6.6 Hz, 3H, 8Me), 1.04 (d, J = 7.2 Hz, 3H, 4Me), 1.06 (d, J = 7.0 Hz, 3H, 6Me), 1.09 (d, J = 7.0 Hz, 3H, 2Me), 1.13 (d, J = 6.1 Hz, 3H, 5′Me), 1.18 (m, 1H, 4′ax), 1.19 (m, J = 5.9 Hz, 3H, 5″Me), 1.20 (d, J = 6.3 Hz, 3H, 14), 1.27 (m, 3H, 7a), 1.44 (ddd, J = 12.9, 11.0, 4.0 Hz, 1H, 2″ax), 1.45 (m, 1H, 12), 1.50 (quin, J = 7.3 Hz, 1H, 10), 1.58 (m, 1H, 8), 1.62 (m, 1H, 4), 1.63 (m, 1H, 6), 1.64 (ddd, J = 12.9, 4.0, 1.9 Hz, 1H, 4′eq), 2.17 (ddd, J = 12.9, 4.5, 1.4 Hz, 1H, 2″eq), 2.27 (s, 6 H, 3′NMe), 2.54 (ddd, J = 13.3, 9.2, 3.5 Hz, 1H, 3′ax), 2.70 (quin, J = 7.1 Hz, 1H, 2), 2.94 (td, J = 8.7, 5.1 Hz, 1H, 4″ax), 3.11 (dq, J = 9.9, 7.5 Hz, 1H, 2′ax), 3.14 (t, J = 7.9 Hz, 1H, 9), 3.19 (t, J = 8.0 Hz, 1H, 11), 3.35 (s, 3H, 3″OMe), 3.39 (m, 1H, 3″eq), 3.41 (m, 1H, 5), 3.54 (dq, J = 10.1, 6.1 Hz, 1H, 5′ax), 3.57 (d, J = 7.3 Hz, 1H, 3), 3.64 (dq, J = 9.1, 6.1 Hz, 1H, 5″ax), 3.71 (d, J = 5.6 Hz, 1H, 9OH), 3.88 (br s, 1H, 2′OH), 4.21 (d, J = 6.8 Hz, 1H, 1′ax), 4.79 (d, J = 5.1 Hz, 1H, 4″OH), 4.89 (d, J = 2.4 Hz, 1H, 1″eq), 5.36 (q, J = 6.5 Hz, 1H, 13) ppm; ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C): δ = 8.0 (12Me), 8.8 (4Me, 10Me), 13.3 (2Me), 17.4 (5″Me), 17.9 (14), 18.5 (8Me), 18.6 (6Me), 20.5 (5′Me), 29.0 (7), 30.0 (4′), 33.1 (6), 34.0 (2″), 36.0 (8), 36.8 (10), 39.8 (3′NMe), 41.2 (4), 42.2 (12), 43.5 (2), 56.1 (3″OMe), 64.5 (3′), 67.5 (5′), 68.7 (5″), 69.2 (11), 69.5 (13), 70.0 (2′), 75.4 (4″), 77.0 (3″), 77.9 (9), 80.0 (3), 82.4 (5), 97.9 (1″), 103.6 (1′), 175.0 (1) ppm. HRMS-ES m/z [M+H]+ calcd for $C_{35}H_{66}NO_{11}$: 676.4636, found: 676.4625.

4.4. Antimicrobial activity

Whole-cell antimicrobial activity of compounds against macrolide susceptible Gram-positive (*Staphylococcus aureus* (ATCC 13709), *Streptococcus pneumoniae* (ATCC49619), *Streptococcus pyogenes* (ATCC700294), and Gram-negative (*Moraxella catarrhalis* (ATCC23246), and *Haemophilus influenzae* (ATCC49247) bacterial strains was determined by broth microdilution test using the Clinical and Laboratory Standards (CLSI) recommended procedure (Document M7-A6A7, Methods for Dilution Susceptibility Tests for Bacteria that Grow Aerobically). The minimum inhibitory concentration (MIC) is determined as the lowest concentration of compound that inhibited visible growth.

4.5. LPS-induced IL-6 production by murine splenocytes

After cervical dislocation, mouse spleens were removed using sterile dissection tools. Spleens were transferred to a pre-wetted cell strainer in a 50 ml sterile conical tube and cell suspension was made by gentle puddle. Cells were centrifuged (20 min, 300 g) and resuspended in 2 ml of sterile phosphate buffered saline (PBS) (Sigma Chemical Co., USA). Red blood cells were lysed by addition of 3 ml of sterile water and occasionally gentle shaking for 1 min. Afterwards, the tube was filled to 40 ml with DMEM medium and centrifuged (20 min, 300 g). Cells were resuspended in DMEM supplemented with 1% FBS and seeded in a 24-well plate, 10^6 cells per ml medium. Cells were pre-incubated with the test compounds for 2 h at 37 °C, in an atmosphere of 5% CO_2 and 90% humidity. Afterwards, cells were stimulated with 1 μg/ml lipopolysaccharide (LPS, *E. coli* 0111:B4, Sigma Chemical Co., USA) and incubated overnight. Concentration of IL-6 was determined in cell supernatants by sandwich ELISA using capture and detection antibodies (R&D Systems, USA) according to the manufacturer's recommendations. Inhibition (as percentage) was calculated using the following formula:

$$\%\text{inhibition} = [1 - (\text{concentration of IL-6 in sample} - \text{concentration of IL-6 in negative control})/(\text{concentration of IL-6 in positive control} - \text{concentration of IL-6 in negative control})] \times 100$$

The positive control refers to LPS-stimulated samples that were not preincubated with the compounds. The negative control refers to unstimulated and untreated samples.

4.6. Cell viability

THP-1 cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum at 37 °C in the presence of test compounds at concentrations 100–0.8 μM for 24 h. Afterwards, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] (Promega, US), a detection reagent, was added and cells were incubated for additional 0.5–2 h. The amount of MTS-formazan produced was determined using a spectrophotometer at 490 nm. Percentage of inhibition of cell viability was calculated using the following formula:

$$\%\text{inhibition of cell viability} = \text{OD}_{490}\text{treated cells}/\text{OD}_{490}\text{untreated cells} \times 100$$

4.7. LPS-induced lung neutrophilia

Studies were performed on 10 week old male BALB/cJ mice (Charles River, Lyon, France). Mice were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Settimo Milanese, Italy) and maintained under standard laboratory conditions (temperature 23–24 °C, relative humidity 60 ± 5%, 15 air changes per hour, artificial lighting with circadian cycle of 12 h. Pelleted food (VRF-1, Altromin, Charles River, Isaszag, Hungary) and tap water were provided ad libitum.

All procedures on animals were approved by the ethics committee of GlaxoSmithKline Research Centre Zagreb Limited, and performed in accordance with the European Economic Community Council Directive 86/609.

Briefly, mice, under light anesthesia, were instilled intranasally with 2 μg LPS from *E. coli*/60 μl PBS. Vehicle or compounds were administered intraperitonealy 2 h before intranasal challenge with LPS at a dose of 100 mg/kg (bw). For administration, macrolides were first dissolved in dimethlysulfoxide (DMSO) and then diluted with 0.5% (w/v) methyl-cellulose [final concentration of DMSO was 5% (v/v)]. Solutions obtained were applied in a volume of 20 ml/kg (bw).

The animals were euthanized by an intraperitoneal overdose of Thiopental® (Inresa Arzneimittel GmbH, Freiburg, Germany) 24 h after LPS application After preparation and cannulation of tracheas, bronchoalveolar lavage was performed with phosphate buffered saline (PBS) in a total volume of 1 ml (0.4, 0.3 and 0.3 ml). The bronchoalveolar lavage samples were centrifuged (4 °C, 100 g, 5 min), the pellet of cells resuspended in an equal volume of fresh PBS and used for total and differential cell counts. Total number of cells in BALF was counted with a hematological analyzer (Sysmex SF 3000, Sysmex Co., Kobe, Japan). Percentages of neutrophils were determined by morphological examination of at least 200 cells on smears prepared by cytocentrifugation (Cytospin-3, Thermo Fisher Scientific Inc., Pittsburgh, PA, US) and stained with Kwik-Diff staining set (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Number of neutrophils (and macrophages) in BALF was calculated for each sample according to the formula:

$$\text{number of neutrophils} = \text{total number of cells} \times (\text{neutrophil percentage}/100\%)$$

4.8. Statistical analysis

All values are presented as means ± SEM. To define statistically significant differences in cell numbers between vehicle-treated and macrolide-treated mice 24 h after LPS challenge, the data were subjected to Kruskal-Wallis test followed by Dunn's multiple comparison test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, US). The level of significance was set at $p < 0.05$ in all cases.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.02.013.

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