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First Synthesis of Racemic Saphenamycin and Its Enantiomers. Investigation of Biological Activity†

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Abstract—The natural antibiotic saphenamycin, 6-[1-(2-hydroxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid, was synthesized from saphenic acid using temporary allyl protection of carboxy and phenoxy functionalities. Resolution of racemic saphenic acid was performed by crystallization of the corresponding (−)-brucine diastereomeric salts and the absolute configuration of (−)-brucinium (−)-saphenate was determined by X-ray crystallography to have *R*-configuration. This also proved to be the configuration of natural saphenic acid. Enantiomers of saphenamycin were obtained from resolved saphenic acid and screened against a range of skin flora and resistant *Staphylococcus aureus* strains. Biological activities of saphenamycin enantiomers were compared with that of the synthetic racemate as well as earlier reported activities of saphenamycin isolated from natural sources. No significant difference was observed in activity of the enantiomers of saphenamycin, which revealed that the chirality of saphenamycin has no consequences for the antibiotic activity. Saphenamycin proved to be a potent antibiotic against fusidic acid and rifampicin resistant *S. aureus* strains showing MIC of 0.1–0.2 μg/mL. ©2002 Elsevier Science. All rights reserved

Introduction

Bacteria and fungi synthesize a plethora of biologically active natural compounds with functional characteristics such as antibiotic and antitumor activity. Several of these compounds are believed to act directly on nucleic acids modulating replication, transcription or translation processes.[1–4]

Among these compounds is the yellow pigment and secondary metabolite saphenamycin (**1**), 6-[1-(2-hydroxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid (Fig. 1), which was isolated from a strain of *Streptomyces* by Michel et al. in the early 1980s and later also by other research groups.[5–7] Saphenamycin was found to exhibit a broad range of biological activities including antibacterial activity against Gram-positive and Gram-negative bacteria,[5,6,8] antitumor activity,[6,8] antitrichomonal activity,[5] and larvacidal activity against mosquitoes.[5]

Saphenamycin contains the flat tricyclic aromatic phenazine chromophore, which has been found in several other secondary metabolites isolated from various bacterial genera.[9] Many of these are known to be antibiotics and virulence factors.[9] Recently, we reported the synthesis of new analogues closely related to saphenamycin. One analogue exhibited activity against several bacteria with potency similar to saphenamycin.[10]

The antibiotic action of phenazines and other planar aromatic molecules may be complex but intercalation

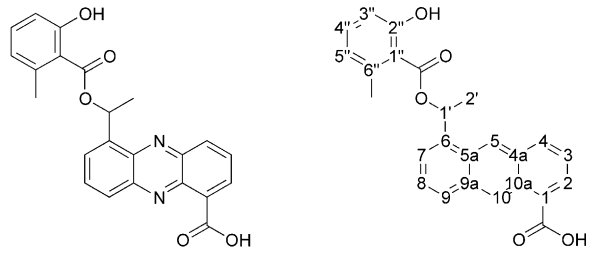

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Figure 1. Structure and general atom numbering of saphenamycin (**1**).

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into duplex DNA has been proposed for several phenazine antibiotics.[11,12] Also, generation of toxic anionic radicals involved in redox cycling has been suggested as a mode-of-action for phenazine-based antibiotics.[9]

Saphenamycin contains one asymmetric carbon atom, the secondary benzylic position (C1′, Fig. 1). Early X-ray crystallographic studies performed on saphenamycin isolated from natural sources by Kitahara et al.[6] revealed only the relative structure of saphenamycin and specific rotations of isolated samples were close to 0 and very inconsistent.[5,6,8] Until now, no reports have appeared on the absolute configuration of naturally occurring saphenamycin.

The present paper reports an efficient synthesis of saphenamycin as a racemate using allyl protection. Furthermore, the successful resolution of saphenic acid and elucidation of its absolute configuration has led us to the synthesis, identification and biological testing of the enantiomers of saphenamycin. To the best of our knowledge this is the first report on elucidation of the absolute configuration of these naturally occurring phenazine derivatives.

Synthetic strategy

Simple disconnection of the ester bond in saphenamycin gives two moieties; 6-methylsalicylic acid (**2**) and saphenic acid (**3**). When appropriately protected these moieties will couple to form a protected form of saphenamycin. Allyl protection proved ideal for accomplishment of the synthesis.

Results and Discussion

Synthesis of 6-methylsalicylic acid (2) and racemic saphenic acid (3)

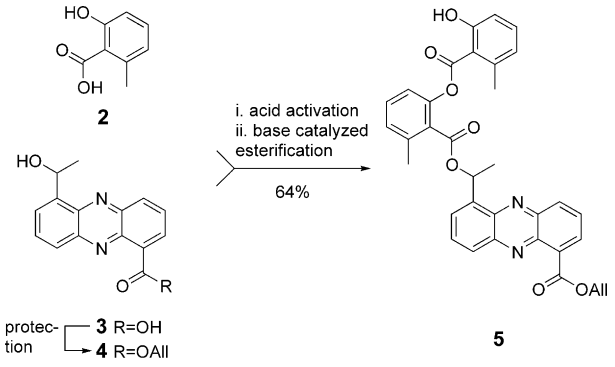
Several methods for preparing 6-methylsalicylic acid (**2**) have been reported. The method described by Hauser et al. in 1980[13] provided 6-methylsalicylic acid ethyl ester (**6**), the first starting material in our saphenamycin synthesis. The Hauser method involved a Robinson annulation reaction of inexpensive starting materials, crotonic acid and ethyl acetoacetate. Bromination followed by dehydrobromination gave 6-methylsalicylic acid ethyl ester (**6**) in a few moderate to high yielding steps.

In 1999, we reported an improved synthesis of racemic saphenic acid (**3**) using a non-stereoselective reductive ring closing method.[14] This provided us with the second starting material for racemic saphenamycin synthesis.

Synthesis of racemic saphenamycin (1)

Early attempts by Bahnmüller et al. to synthesize saphenamycin from unprotected saphenic acid (**3**) and protected as well as unprotected 6-methylsalicylic acid failed.[15] The lack of success could be ascribed to inadequate reactivity of the sterically hindered 6-methylsalicylic acid derivatives towards thionyl chloride. In our hands, preparation of the sterically hindered acid chlorides, unprotected as well as methyl- and allyl-protected, proceeded smoothly under 'Vilsmeier'-conditions using oxalyl chloride in the presence of a catalytic amount of DMF.[16]

Protection of both saphenic acid (**3**) and 6-methylsalicylic acid (**2**) seemed crucial for the synthesis in order to minimize formation of byproducts. Esterification of allyl-protected saphenic acid (**4**) with excess of unprotected 6-methylsalicylic acid gave a 'double ester' **5** of saphenamycin in 64% yield and only limited amounts of the desired partially protected saphenamycin (Scheme 1).[†]



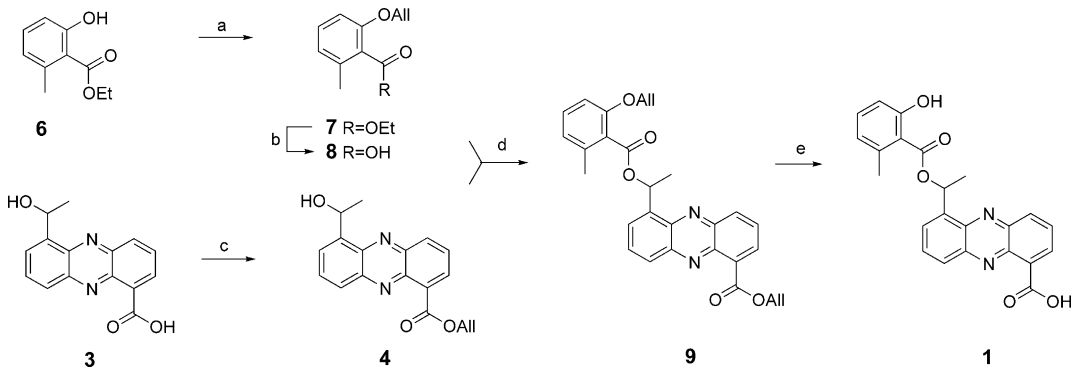
Scheme 1. Formation of 'double ester' byproduct when esterifying allyl saphenate (**4**) with excess 6-methylsalicylic acid (**2**).

Moreover, adding unprotected saphenic acid (**3**) to *o*-toluic acid chloride resulted in several byproducts. Use of identical protecting groups for phenol and carboxylic acid reduced the number of deprotection steps. Furthermore, earlier reports had drawn attention to steric hindrance of the *ortho*-substituted benzoic acid (vide supra). This emphasizes use of a sterically less demanding protecting group for the phenol that is essential for satisfactory reactivity of the acid. Additionally, orthogonality of protecting groups and the benzylic ester in saphenamycin was essential. Thus, the small and easily removable allyl group[17] was utilized for protection of hydroxyl as well as carboxyl functionalities in the synthesis of saphenamycin (**1**).

Saphenic acid was protected as the allyl ester (**4**) in quantitative yield using a method described for amino acid derivatives.[18] Allyl-protected 6-methylsalicylic acid, 2-allyloxy-6-methylbenzoic acid (**8**), was obtained from a Williamson allylation of 6-methylsalicylic acid ethyl ester (**6**) followed by hydrolysis of the ethyl ester (**7**) in 92% yield for the two steps (Scheme 2).

O-Allyl-6-methylsalicylic acid (**8**) was treated with oxalyl chloride and DMF, forming the acid chloride quantitatively. The presence of the corresponding acid chloride was confirmed by NMR, which showed a change in chemical shift of the 6-methyl group from 2.46 to 2.38 ppm in agreement with reported NMR data

[†]Limiting the excess of **2** (from 4 to 2 equiv) led to formation of several byproducts and even lower yield of the desired product.



Scheme 2. Synthesis of saphenamycin; (a) All-Br, K_2CO_3, acetone, reflux 24 h (94%); (b) NaOH (s), EtOH/H_2O, reflux, 30 h (98%); (c) All-Br, DIEA, CH_3CN, rt 18 h (quant.); (d) (1) $(COCl)_2$/DMF, CH_2Cl_2, 2 h; (2) DMAP, pyridine, room temp. 24 h. (64%); (e) Et_2NH, $Pd^0(PPh_3)_4$ (cat.), dioxane, rt 2.5 h. (88%). 88% of the *R*-isomer was obtained using optimized conditions as listed in this scheme. The racemic saphenamycin was prepared using non-optimized conditions that yielded 61%.

of *O*-methyl-6-methylsalicylic acid and the corresponding acid chloride.[19] Furthermore, one drop of the reaction mixture was treated with methanol and the methyl ester was visualized by TLC analysis. The acid chloride was concentrated in vacuo and used directly without further purification.

Conditions for the base-catalyzed esterification of saphenic acid allyl ester (**4**) and *O*-allyl-6-methylsalicylic acid chloride were investigated. Reaction in CH_2Cl_2 with DMAP as a catalyst and Et_3N as stoichiometric base was sluggish. More polar conditions using DMAP as a catalyst and pyridine as both solvent and base afforded the allyl-protected saphenamycin (**9**) in good yield (64%).

Deallylation of compound **9** relied on Pd^0 catalysis via formation of a presumed π-allyl palladium(II) complex. Regeneration of Pd^0 can be achieved by either hydride donation or cleavage of the allyl palladium species by addition of another nucleophile. First, $Pd(PPh_3)_4$ was used in catalytic amount as palladium source and the use of several conventional external hydride donors ($NaBH_4$, Bu_3SnH and $PhSiH_3$) was investigated, but all reactions failed. This may be ascribed to the lack of free coordination sites on the palladium source used. Next, internal hydride donation from formate with triethylamine as base was investigated, and in this case the allyl ester was readily cleaved, while conversion of the allyl ether was sluggish. Cleavage of the allyl ester creates the nucleophilic carboxylate that competes with formate in the coordination to bis(triphenylphosphine)-palladium (II). However, it is still likely that the allyl ester is cleaved without palladium catalysis by direct transesterification to form the allylformate. Saphenate carboxylate coordination may hinder coordination of formate, hence the intramolecular hydride delivery from formate to Pd^{II} and locks the catalyst in its oxidized state inhibiting the catalytic cycle. Finally, an external nucleophile was added to successfully trap the Pd^{II}-bound allyl and regenerate Pd^0 without use of additional coordination sites on palladium. In our hands, separation of saphenamycin (**1**) from the corresponding aryl allyl ether proved to be particularly difficult. This demonstrated the importance of full conversion in the deprotection step in order to obtain acceptable yields. Using 2.25 equiv of a simple secondary amine, diethylamine, and 0.15 equiv of Pd^0 full deprotection and up to 88% isolated yield was obtained.

Characterization of compounds

The phenazine compounds were characterized by MS and NMR and a full assignment is included here to resolve contradictions in earlier reported assignments.[7,20,21] The relative assignment within the groups of protons 2–4 and 7–9 could be established by COSY spectroscopy. The complete assignment of protons on the phenazine ring was achieved using gHSQC and gHMBC spectroscopy. Especially, the 3-bond coupling between the carboxyl-C and H-2 was useful.

It was observed that esterification of the 1′-OH with protected 6-methyl salicylic acid greatly affected the shift of H-1′ (from 5.7 to 7.5 ppm). Moreover, the H-2 shift changed dramatically (from 8.3 to 9.0 ppm) upon deprotection of **9** to saphenamycin (**1**) (Table 1).

The assignment of H-2 and H-4 in allyl saphenate (**4**) is in agreement with the assignment of methyl saphenate made by Van't Land[20] and Yun[21] but it differs from the assignment of these protons in methyl saphenate made by Keller-Schierlein et al.[7]

Resolution of saphenic acid (3)

With racemic saphenic acid already at hand,[14] effort was focused on resolution of racemic saphenic acid rather than on developing a stereoselective synthesis. Initial experiments with separation of the enantiomers of saphenamycin (**1**) and saphenic acid (**3**) on semi-preparative chiral HPLC provided us with small analytical samples. The amounts of **1**, however, were inadequate to fully characterize and analyze the enantiomers of the antibiotic, and the amounts of **3** inadequate to perform further synthesis in order to obtain the enantiomers of saphenamycin. Hence, methods for resolution of saphenic acid on larger scale were considered.

Table 1. ^{1}H NMR data (ppm) in $CDCl_3$[a]

Compound	H-2	H-3	H-4	H-7	H-8	H-9	H-1′	H-2′	H-3″	H-4″	H-5″	H-7″
6									6.84	7.26	6.71	2.55
$^3J_{(H,H)}$ Hz									8.0	7.6, 8.2	7.7	
7									6.74	7.19	6.79	2.30
$^3J_{(H,H)}$ Hz									8.5	8.0, 8.0	7.5	
8									6.82	7.28	6.88	2.48
$^3J_{(H,H)}$ Hz									8.4	7.5, 8.4	7.5	
4	8.26	7.77–7.88	8.34	7.77–7.88	7.77–7.88	8.20	5.71	1.79				
$^3J_{(H,H)}$ Hz	6.9	m	8.7	m	m	8.3	6.6	6.7				
9	8.28	7.85	8.46	8.04	7.85	8.25	7.51	1.87	6.78	7.24	6.82	2.32
$^3J_{(H,H)}$ Hz	6.8	m	9.0	6.5	m	9.0	6.8	6.5	8.5	8.1, 8.5	7.7	
1	9.02	8.08	8.61	8.01	8.01	8.26	7.49	1.97	6.85	7.31	6.77	2.73
$^3J_{(H,H)}$ Hz	7.0	7.0, 8.8	8.8	m	m	6.6	6.3–6.6	6.5	8.4	7.3, 8.4	7.7	

[a]Assignment in accordance to gHSQC, gHMBC and H,H-COSY experiments (*m* = multiplet).

Formation of saphenic acid derived Mosher's esters was accomplished in excellent yields but separation of the diastereomeric esters failed by normal-phase as well as reversed-phase chromatography.

Finally, our attention was drawn to resolution using (−)-brucine, which had received substantial attention in literature.[22,23] Resolution of saphenic acid was completed by (−)-brucine salt formation and crystallization from methanol. The free acids were liberated by treatment with NH_3 (25% aq solution) and isolated by acidification with HCl followed by concentration. After the first crystallization, enantiomeric purity of the acid of up to 80% was obtained in high yield. Successive crystallizations only improved the enantiomeric purity slightly, but also lowered the recovered yield of both enantiomers considerably. Attempts to directly resolve saphenamycin by brucine salt formation were unsuccessful.

The brucine salt precipitated from the resolution was recrystallized from water/methanol and plate crystals were obtained and analyzed by X-ray crystallography (Fig. 2).

The absolute configuration of the precipitated (−)-brucinium (−)-saphenate was determined to be the *R*-form and (−)-saphenic acid had a specific rotation of −19.7 (*c*0.13) and +42.0 (*c*0.23) in chloroform and DMSO, respectively. Geiger et al. reported a specific rotation of saphenic acid isolated from a *Streptomyces* strain to be −11.4 (*c*0.57) and +55 (*c*0.16) in chloroform and DMSO, respectively.[8] The small rotations can be tedious to measure in dilute samples and increased concentration leads to high absorption at the wavelength of the sodium-D line. This can explain the differences in rotation determined and it can be concluded that saphenic acid isolated from *Streptomyces* is the (*R*)-enantiomer.

Synthesis of saphenamycin enantiomers

The resolved enantiomers of saphenic acid were carried through the synthesis identical to the racemate (Scheme 2) and similar yields were obtained of the enantiomers of saphenamycin. Enantiopurity was retained throughout the synthesis and no racemization was observed. All intermediates and products were analyzed by chiral HPLC and polarimetry. Table 2 shows a summary of these results.

As can be seen from Table 2, a significant specific rotation of more than 150 in chloroform was determined for both enantiomers of saphenamycin. Earlier reports on optical rotation of saphenamycin samples isolated from natural sources range from 0 to +5 in chloroform.[5,6,8] Partial to complete racemization during isolation, or the existence of racemic saphenamycin in Nature could explain this phenomena. The former is unlikely, since no racemization was detected under reaction conditions ranging from acidic (4 M HCl) to basic (pyridine, DMAP, Et_2NH, aq $NaHCO_3$). The latter is questionable as well, since the biosynthesis of saphenamycin is believed to proceed via shikimic acid to the prochiral

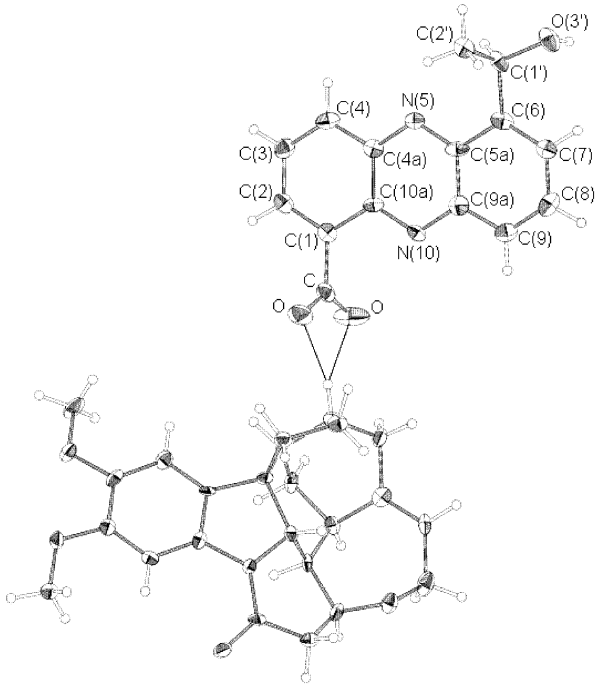


Figure 2. X-ray crystal structure of (−)-brucinium (−)-(*R*)-saphenate. Ellipsoides are 50% probability levels.[24] Hydrogen bonds between carboxylate of saphenic acid and ammonium salt of brucine are marked with thin lines and are 1.9 and 2.3 Å, respectively. Refinements were performed in SHELXL98 and the picture is created by ORTEP II.

Table 2. Polarimetric results, melting points and retention times of enantiomers during saphenamycin synthesis[a]

Compound	Conformation	$[\alpha]_D^{22}$[b]	Melting point (°C), (*R*/*S*)	Retention time (min.)[c]
3	R	−19.7	204–206, (83:17)	28.8
	S	+20.0	203–205, (10:90)	33.0
4	R	−27.3	99–101, (69:31)	11.2
	S	+34.3	102–104, (21:79)	12.7
9	R	−53.5	Oil, (81:19)	13.2
	S	+50.0	Oil, (16:84)	14.9
1	R	−150.6	159–164, (90:10)	54.2
	S	+176.9	162–165, (19:81)	49.2

[a]Specific rotations of enantiomeric pairs are not numerically identical. This is ascribed to the small amounts weighed, since high concentrations resulted in poor transmittance at 589 nm.
[b]Measured in chloroform using the sodium-D line (589 nm). Corrected according to enantiopurity (see Experimental).
[c]HPLC on Chiralcel OD-H column eluting with hexane/2-propanol/TFA (70:30:0.1).

ketone 6-aceto-phenazine-1-carboxylic acid.[20] A subsequent enzymatic reduction, which is presumably enantioselective, leads to formation of saphenic acid. Successive esterification to form saphenamycin is not likely to deteriorate optical activity. Simple esterification of the free carboxylic acid in saphenamycin isolated from natural sources yields esters with significant specific rotation (>200 in chloroform).[8] These arguments indicate that saphenamycin in Nature is chiral and is not likely to racemize under extraction and isolation conditions. Hence, earlier reports on the specific rotation appear questionable, and our measurements cannot reveal the absolute configuration of the naturally occurring species. Saphenic acid has now been identified as the *R*-form, and it is therefore plausible that saphenamycin in Nature is also the *R*-form.

Biological activity

The importance of chirality for antimicrobial activity of saphenamycin can be crucial information in determination of mode-of-action of this class of antibiotics. Several reports on biological activities of saphenamycin samples isolated from natural sources have appeared.[5–8] These can be directly compared with our observations of synthetic racemate as well as enantiomers.

Growth inhibition studies were performed on a panel of Gram-positive skin flora and on two resistant *Staphylococcus aureus* strains; clinical isolates of fusidic acid resistant and rifampicin resistant MRSA (Table 3). The zones of growth inhibition were sharp and clear indicating bactericidal and not bacteristatic character of the antibiotics. No significant difference was detected between activities of enantiomers and racemate of saphenamycin against any organism, indicating that the stereochemistry had no influence on activity. In all cases, the antibiotic activity of saphenamycin was stronger or equivalent to that of ceftriaxone and ciprofloxacin. MIC values ranged from <0.1 to 0.3 μg/mL and were comparable to the values reported for natural isolates.[5,6]

Conclusions

The first reported synthesis of the natural antibiotic saphenamycin was carried out with success starting from synthetic saphenic acid and 6-methylsalicylic acid. The racemate was synthesized with satisfactory yields using a temporary allyl protection of carboxy and phenoxy functionalities from racemic saphenic acid.

Racemic saphenic acid was successfully resolved by crystallization of the corresponding (−)-brucine diastereomeric salts and the absolute configuration of (−)-brucinium (−)-saphenate was determined by X-ray crystallography to have *R*-configuration. Based on earlier reported polarimetry analysis, we suggest that

Table 3. Antibacterial activity of Saphenamycin racemate and enantiomers[a]

Organism/strain	MIC (μg/mL)				
	Natural **1**	(±)-**1**	(−)-(*R*)-**1**[c]	(+)-(*S*)-**1**[c]	Reference[d]
Micrococcus luteus ATCC 9341	0.01–0.39[b,6]	0.01	0.01	0.04	<0.01
Propionibacterium acnes ATCC 6919	0.015–2[b,5]	0.01	0.02	0.02	0.64
Streptococcus pyogenes NCTC 8304	n.a.	0.03	0.04	0.06	<0.001
Streptococcus pyogenes ATCC 10403	n.a.	0.19	0.30	0.30	1.84
Staphylococcus epidermidis ATCC 12228	n.a.	0.05	0.16	0.12	0.76
Staphylococcus aureus ATCC 6538P, FDA 209P	0.39[6]	0.11	0.17	0.17	0.10
Staphylococcus aureus ATCC 25923	n.a.	0.20	0.16	0.22	0.18
Staphylococcus aureus CJ 234 (F) (MRSA)	n.a.	0.09	0.16	0.26	0.24
Staphylococcus aureus CJ 234 (R) (MRSA)	n.a.	0.11	0.11	0.18	0.32

[a]MRSA, meticilline resistant *S. aureus*; F, fusidic acid resistant clinical isolate; R, rifampicin resistant clinical isolate; n.a., not available.
[b]MIC reported in literature on similar genera but not identical strains.
[c]Enantiopurity: (−)-(*R*)-**1** (81% *R*, 19% *S*), (+)-(*S*)-**1** (21% *R*, 79% *S*).
[d]Ceftriaxone (susceptible strains), ciprofloxacin (resistant strains).

this enantiomer is the naturally occurring stereoisomer of saphenic acid. The enantiomers of saphenamycin were synthesized from the corresponding saphenic acid enantiomers, also with satisfactory yields.

Optical studies of earlier isolated samples of saphenamycin have been inconsistent. Based on the knowledge of the biosynthetic pathway and on determination of significant specific rotations of the synthetic enantiomers, it is believed that saphenamycin in Nature exists as one enantiomer. Thus, we suggest that the natural configuration of this enantiomer is equivalent to its parent saphenic acid, namely the *R*-form.

The enantiomers of saphenamycin were screened against a range of normal skin flora and resistant *S. aureus* strains. These were compared to the synthetic racemate as well as earlier reported biological activities of saphenamycin isolated from natural sources. No significant difference was observed in the antibacterial activity of the enantiomers of saphenamycin, thus it can be concluded that the chirality of saphenamycin is of less importance to its biological activity. Saphenamycin proved to be a potent antibiotic against fusidic acid and rifampicin resistant *S. aureus* strains showing MIC values between 0.1 and 0.2 μg/mL.

Further studies in our laboratories will elucidate the mode-of-action of this class of molecules. Different structural variations in order to improve activity or even enhance specificity will be investigated.

Experimental

Commercially available reagents were used without further purification unless otherwise noted. Saphenic acid[14] and 6-methylsalicylic acid ethyl ester[13] were prepared as previously reported. Solvents for reactions were distilled prior to use and stored over molecular sieves. Flash chromatography was performed manually on silica gel (Matrex 60 Å, 35–70 μ) or automatically on a Biotage Quad3+ apparatus with prepacked columns (KP-SIL, 32–63 μm, 60 Å). Thin layer chromatography was performed on Merck silica gel 60 F_{254} alumina plates. Corrected melting points were measured in open capillary tubes. Analytical HPLC was performed using Merck Hitachi LaChrom system (pump L-7100, autosampler L-7200, column oven L-7300, diode array detector L-7450) with a Supercosil RP-3,3 column. Gradients of 0.035% TFA in CH_3CN (A) and 0.05% aqueous TFA (B) with a flow of 1.5 mL/min were used as follows: 100% B for 30 s; from 100% B to 100% A over 10 min; 100% A for 2 min; from 100% A to 100% B over 6 s; 100% B for 2 min. Preparative HPLC was performed on a Waters 600 system with Waters 996 diode array detector and three consecutive columns (40×100 mm prep. NOVA Pak HR C18 6 μm 60 Å units). Linear gradients of CH_3CN and water (MilliQ) were used. Chiral HPLC was performed on a Chiralcel OD-H column (0.46 cm Ø×25 cm) eluting with hexane/2-propanol/TFA (70:30:0.1) in a Varian 9012 Solvent Delivery System and visualized at 254 nm by Varian 9065 Polychrom UV detector. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC200, a Varian Mercury 300 or a Varian Unity Inova 500 spectrometer. The chemical shifts are referred to an internal standard of $CDCl_3$. gHMBC and H,H-COSY spectroscopy were recorded on a Varian Mercury 300 apparatus. Mass spectrometry was performed on a VG Masslab trio-2 apparatus (EI–MS) and accurate mass determination (high resolution MS, HR-MS) was performed on a Micromass LCT apparatus with an AP-ESI probe.

2-Allyloxy-6-methylbenzoic acid ethyl ester (7). A mixture of 2-hydroxy-6-methylbenzoic acid ethyl ester (**6**) (9.0 g, 50 mmol, 1 equiv), K_2CO_3 (8.3 g, 60 mmol, 1.2 equiv), allyl bromide (8.5 mL, 100 mmol, 2 equiv) and acetone (70 mL) was refluxed for 24 h. The mixture was concentrated in vacuo, diluted with EtOAc, washed with H_2O and brine, dried with Na_2SO_4 and concentrated to give the title compound **7** as a yellow oil (10.3 g, 94%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.37 (t, $J=7.2$ Hz, $C\underline{H}_3CH_2$, 3H), 2.30 (s, H-7″, 3H), 4.39 (q, $J=7.2$ Hz, $CH\underline{H}_2CH_3$, 2H), 4.55 (dt, $J=1.4$–1.9, 5.0 Hz, $OC\underline{H}_2CH=C\underline{H}_2$, 2H), 5.24 (dd, $J_{gem}=1.4$, $J_{cis}=10.6$ Hz, $CH=C\underline{H}_2$, 1H), 5.38 (dd, $J_{gem}=1.7$ Hz, $J_{trans}=17.2$, $CH=C\underline{H}_2$, 1H), 5.95-6.05 (m, 1H), 6.74 (d, $J=8.5$ Hz, H-3″, 1H), 6.79 (d, $J=7.5$ Hz, H-5″, 1H), 7.19 (t, $J=8.0$ Hz, H-3‘, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.6 ($\underline{C}H_3CH_2$), 19.4 (C-7″), 61.2 ($\underline{C}H_2CH_3$), 69.5 ($O\underline{C}H_2CH=CH_2$), 110.2 (C-3″), 117.3 ($\underline{C}H_2=CH$), 122.8 (C-5″), 124.8 (C-1″), 130.2 (C-4″), 133.2 ($\underline{C}H=CH_2$), 136.6 (C-6″), 155.6 (C-2″), 168.4 (C=O) ppm. EI–MS calcd m/z: 220, found 220 $[M]^+$.

2-Allyloxy-6-methylbenzoic acid (8). A homogeneous mixture of 2-allyloxy-6-methylbenzoic acid ethyl ester (**7**) (7.7 g, 35 mmol, 1 equiv), NaOH (2.8 g, 70 mmol, 2 equiv), H_2O (20 mL) and EtOH (20 mL) was refluxed for 30 h. After 2 h at reflux the mixture shifted from yellow to a brown turbid color and after a total of 30 h at reflux it had turned into a clear yellow color. The mixture was concentrated, acidified using concentrated HCl (aq) and extracted with EtOAc (3×25 mL). The combined organic phases were concentrated in vacuo yielding the product **8** as a yellow oil (6.6 g, 98%). ^1H NMR (300 MHz, $CDCl_3$) δ 2.48 (s, H-7″, 3H), 4.64 (dt, $J=1.5$, 5.1 Hz, $OC\underline{H}_2CH=CH_2$, 2H), 5.31 (dd, $J_{gem}=1.2$–1.5, $J_{cis}=10.4$ Hz, $CH=C\underline{H}_2$, 1H), 5.44 (dd, $J_{gem}=1.3$–1.8 Hz, $J_{trans}=17.2$, $CH=C\underline{H}_2$, 1H), 5.98–6.09 (m, 1H), 6.82 (d, $J=8.4$ Hz, H-3″, 1H), 6.88 (d, $J=7.5$ Hz, H-5″, 1H), 7.28 (dd, $J=7.5$, 8.4 Hz, H-4″, 1H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.2 (C-7″), 69.7 ($O\underline{C}H_2CH=CH_2$), 110.2 (C-3″), 117.9 ($\underline{C}H_2=CH$), 121.9 (C-1″), 123.5 (C-5″), 131.0 (C-4″), 132.4 ($\underline{C}H=CH_2$), 138.6 (C-6″), 155.9 (C-2″), 171.7 (C=O) ppm. EI–MS calcd m/z: 192, found 192 $[M]^+$.

Racemic 6-(1-hydroxy-ethyl)-phenazine-1-carboxylic acid allyl ester, allyl saphenate ((±)-4). To a flask containing racemic saphenic acid (±)-**3** (400 mg, 1.5 mmol, 1 equiv) under argon was added allyl bromide (3 mL, 35.5

mmol, 24 equiv) and CH_3CN (2×1 mL). DIEA (255 μL, 1.5 mmol, 1 equiv) was added dropwise. The mixture was left stirring at ambient temperature under argon for 18 h. The mixture was diluted with EtOAc, washed with 10% Na_2CO_3 (aq, 3×) and brine, dried with $MgSO_4$ and concentrated in vacuo. Flash chromatography (3:2 hexane/EtOAc) gave the product, which was recrystallized from EtOAc/hexane to give (±)-**4** as fine yellow crystals in quantitative yield (460 mg). Mp 87–88 °C. R_f = 0.3 (2:1 hexane/EtOAc). Anal. HPLC (251 nm, >99%, 220 nm, >95%) R_T = 5.71 min. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.79 (d, J = 6.7 Hz, H-2′, 3H), 4.83 (br. s, OH, 1H), 5.04 (dt, J = 1.5, 5.6 Hz, $C\underline{H}_2CH{=}CH_2$, 2H), 5.36 (dq, J_{cis} = 1.4, 10.4 Hz, CH=C$\underline{H}_2$, 1H), 5.60 (dq, J_{trans} = 1.7, 17.2 Hz, CH=C$\underline{H}_2$, 1H), 5.71 (q, J = 6.6 Hz, H-1′, 1H), 6.09–6.21 (m, 6.15, $C\underline{H}{=}CH_2$, 1H), 7.77–7.88 (m, H-3, H-7, H-8, 3H), 8.20 (d, J = 8.3 Hz, H-9, 1H), 8.26 (dd, J = 1.2, 6.9 Hz, H-2, 1H), 8.34 (dd, J = 1.4, 8.7 Hz, H-4, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.9 (C-2′), 66.4 ($O\underline{C}H_2CH{=}CH_2$), 68.9 (C-1′), 118.7 ($OCH_2CH{=}\underline{C}H_2$), 127.4 (C-7), 129.5 (C-3), 129.7 (C-9), 131.0 (C-8), 131.6 (C-1), 132.3 ($OCH_2\underline{C}H{=}CH_2$), 132.4 (C-2), 133.5 (C-4), 140.8 (C-6), 141.0 (C-10a), 142.1 (C-5a), 142.9 (C-4a), 144.3 (C-9a), 166.4 (C=O). HR-MS calcd m/z: 309.1239, found 309.1036 $[M+H]^+$. Elemental analysis for $C_{18}H_{16}N_2O_3$: C, 70.12; H 5.23; N, 9.09. Found: C, 69.96; H, 5.19; N, 9.04.

Racemic 6-{1-[2-(2-hydroxy-6-methyl-benzoyloxy)-6-methyl-benzoyloxy]-ethyl}-phenazine-1-carboxylic acid allyl ester ((±)-5). To a mixture of 6-methylsalicylic acid (**2**) (79 mg, 0.519 mmol, 1 equiv), DMF (two drops), and CH_2Cl_2 (0.5 mL) was added oxalyl chloride (50 μL, 0.571 mmol, 1.1 equiv) dropwise with a syringe at 0 °C under inert conditions. The mixture was left stirring for 2 h and allowed to reach room temperature. A solution of racemic allyl saphenate (±)-**4** (40 mg, 0.130 mmol, 0.25 equiv) and DMAP (19.8 mg, 0.162 mmol, 0.31 equiv) in CH_2Cl_2 (0.5 mL) was added to the 6-methylbenzoic acid chloride, and evolution of HCl (g) was observed. The mixture was left stirring over night under argon at ambient temperature, diluted with CH_2Cl_2, washed with 2 N HCl (aq), H_2O and brine, dried with $MgSO_4$, concentrated in vacuo and purified by automated column chromatography eluting with 100% CH_2Cl_2. The major product was the 'double ester' **5** (48.1 mg, 64%). Anal. HPLC (251 nm, >93%) R_T = 8.27 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.71 (d, J = 6.7 Hz, H-2′, 3H), 2.33 (s, H-7‴, 3H), 2.49 (s, H-7″, 3H), 5.05 (dt, J = 1.2–1.8, 5.5 Hz, $OC\underline{H}_2CH{=}CH_2$, 2H), 5.39 (dq, J_{gem} = 1.2–1.3 Hz, J_{cis} = 10.3 Hz, 1H), 5.63 (dq, J_{gem} = 1.3–2 Hz, J_{trans} = 16.8 Hz, C(O)$OCH_2CH{=}C\underline{H}_2$, 1H), 6.13–6.21, (m (ddt centered at 6.17 ppm), $OCH_2C\underline{H}{=}CH_2$, 1H), 6.24 (d, J = 7.7 Hz, H-3‴, 1H), 6.65 (d, J = 8.4 Hz, H-5‴, 1H), 7.02 (d, J = 8.1 Hz, H-3″ or H-5″, 1H), 7.07 (t, J = 7.8–7.9 Hz, H-4‴, 1H), 7.23 (d, J = 7.8 Hz, H-3″ or H-5″, 1H), 7.42 (t, J = 7.9 Hz, H-4″, 1H), 7.52 (q, J = 6.1–6.6 Hz, H-1′, 1H), 7.68 (dd, J = 7.0, 8.7 Hz, H-8, 1H), 7.81 (d, J = 6.8 Hz, H-7, 1H), 7.85 (dd, J = 7.0, 8.8 Hz, H-3, 1H), 8.09 (dd, J = 1.4, 8.4 Hz, H-9, 1H), 8.26 (dd, J = 1.6, 7.1 Hz, H-2, 1H), 8.39 (dd, J = 1.4, 9.1 Hz, H-4, 1H), 10.87 (s, OH, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.0, 21.7, 24.0, 66.1, 68.9, 110.7, 115.7, 118.4, 120.5, 122.9, 127.0, 127.5, 128.7, 128.8, 130.1, 130.4, 130.7, 130.8, 131.4, 132.0, 133.9, 135.0, 138.6, 139.5, 140.2, 140.8, 141.3, 141.9, 143.1, 147.4, 163.3, 165.7, 166.4, 170.3 ppm. HR-MS calcd m/z: 577.1975, found 577.2192 $[M+H]^+$.

Only small amounts (18.1 mg, 32%) of the allyl ester of saphenamycin were isolated.

Racemic 6-[1-(2-allyloxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid allyl ester ((±)-9). To a mixture of 2-allyloxy-6-methylbenzoic acid (**8**) (250 mg, 1.301 mmol, 1 equiv), DMF (three drops), and CH_2Cl_2 (1 mL) was added oxalyl chloride (120 μL, 1.418 mmol, 1.1 equiv) dropwise with a syringe under inert conditions. The mixture was left stirring for 2 h at ambient temperature and the solvent was evaporated under inert conditions. A solution of racemic allyl saphenate (±)-**4** (200 mg, 0.649 mmol, 0.5 equiv) and DMAP (8 mg, 0.065 mmol, 0.05 equiv) in pyridine (2.5 mL) was added to the 2-allyloxy-6-methylbenzoic acid chloride, and evolution of HCl (g) was observed. The mixture was left stirring over night under argon at ambient temperature. The color changed from yellow to dark brown. The mixture was held over ice water and acidified until pH ~5. The aqueous phase was extracted with EtOAc (3×). The organic phases were combined, dried over $MgSO_4$, concentrated in vacuo and purified by preparative HPLC yielding the product (±)-**9** as a yellow oil (200 mg, 64%). 28% (±)-**4** was recovered. Anal. HPLC (251 nm, >99%, 220 nm, >97%) R_T = 8.01 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.87 (d, J = 6.5 Hz, H-2′, 3H), 2.32 (s, H-7″, 3H), 4.56 (ddd, J = 1.7, 5.1 Hz, $OC\underline{H}_2CH{=}CH_2$, 2H), 5.05 (ddd, J = 1.3–1.7, 5.5 Hz, $C(\underline{O})OC\underline{H}_2CH{=}CH_2$, 2H), 5.20 (dq, J_{gem} = 1.3–1.7 Hz, J_{cis} = 10.7 Hz, $OCH_2CH{=}C\underline{H}_2$, 1H), 5.35 (dq, J_{gem} = 1.7 Hz, J_{trans} = 17.1 Hz, $OCH_2CH{=}C\underline{H}_2$, 1H), 5.37 (dq, J_{gem} = 1.3 Hz, J_{trans} = 10.7 Hz, $C(O)OC\underline{H}_2CH{=}CH_2$, 1H), 5.62 (dq, J_{gem} = 1.3–1.7, J_{trans} = 17.5 Hz, $C(O)OCH_2CH{=}C\underline{H}_2$, 1H), 5.92–6.00 (m, $OCH_2CH{=}CH_2$, 1H), 6.12–6.20 (m, $C(O)OCH_2C\underline{H}{=}CH_2$, 1H), 6.78 (d, J = 8.5 Hz, H-3″, 1H), 6.82 (d, J = 7.7 Hz, H-5″, 1H), 7.24 (dd, J = 8.1, 8.5 Hz, H-4″, 1H), 7.51 (q, J = 6.8 Hz, 1H), 7.85 (m, H-3 and H-8, 2H), 8.04 (d, J = 6.5 Hz, H-7, 1H), 8.25 (d, J = 9.0 Hz, H-9, 1H), 8.28 (d, J = 6.8 Hz, H-2, 1H), 8.46 (d, J = 9.0 Hz, H-4, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 19.9 (C-7″), 22.9 (C-2′), 66.8 ($C(O)O\underline{C}H_2CH{=}CH_2$), 69.7 ($O\underline{C}H_2CH{=}CH_2$), 70.1 (C-1′), 110.6, 118.3 ($O\underline{C}H_2CH{=}\underline{C}H_2$), 119.1 ($C(O)OCH_2CH{=}\underline{C}H_2$), 123.3, 125.2, 127.6, 129.5, 130.5, 130.8, 131.4, 132.0, 132.8, 132.9, 133.6, 134.7, 137.3, 141.4, 141.5, 141.6, 142.7, 144.2, 156.3 (arom. C–O), 167.1 (C(O)O), 168.1 ($\underline{C}(O)OCH_2CH{=}CH_2$) ppm. HR-MS calcd m/z: 483.1920, found 483.1951 $[M+H]^+$.

Racemic 6-[1-(2-hydroxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid ((±)-1). Compound (±)-**9** (49 mg, 102 μmol, 1 equiv) and $Pd(PPh_3)_4$ (17.6 mg, 15.3 μmol, 0.15 equiv) were dissolved in dioxane (1 mL) under argon, a red color evolved. Et_2NH (24 μL, 230 μmol, 2.25 equiv) was added as an external nucleophile. After 3 h the reaction had ceased and the color changed into brown-green. The reaction mixture was diluted with

CH_2Cl_2 and washed with 2 N HCl (aq) (3×), H_2O (3×) and brine. The organic phase was dried with $MgSO_4$, filtered, concentrated in vacuo and purified by automated column chromatography eluting with 100% CH_2Cl_2. **9** and the allyl aryl intermediate 6-[1-(2-allyloxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid (**9a**) were both at $R_f = 0.21$ (100% CH_2Cl_2), but separation was visualized on TLC using CH_2Cl_2/diethyl ether (2:1) [R_f (**9**) = 0.56; R_f (**9a**) = 0.63]. Racemic saphenamycin (±)-**1** was obtained in 61% yield (25 mg) as yellow-green crystals (using non-optimized conditions). Mp 178–180 °C. Anal. HPLC (251 nm, >90%, 220 nm, >84%) $R_T = 7.37$ min. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.97 (d, $J = 6.5$ Hz, H-2′, 3H), 2.73 (s, H-7″, 3H), 6.77 (d, $J = 7.7$ Hz, H-5″, 1H), 6.85 (d, $J = 8.4$ Hz, H-3″, 1H), 7.31 (t, $J = 7.3$, 8.4 Hz, H-4″, 1H), 7.49 (q, $J = 6.3$-6.6 Hz, H-1″, 1H), 8.00–8.01 (m, H-7 and H-8, 2H), 8.08 (dd, $J = 7.0$, 8.8 Hz, H-3, 1H), 8.26 (dd, $J = 3.3$, 6.6 Hz, H-9, 1H), 8.61 (dd, $J = 1.5$, 8.8 Hz, H-4, 1H), 9.02 (dd, $J = 1.5$, 7.0 Hz, H-2, 1H), 11.12 (s, OH, 1H), 15.44 (br. s, COOH, 1H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.4, 24.7, 70.3, 112.5, 116.1, 123.3, 125.1, 127.8, 128.0, 130.6, 133.1, 134.6, 135.6, 137.9, 140.0, 140.2, 141.2, 141.4, 141.5, 142.8, 163.2, 166.0, 170.9 ppm. HR-MS calcd m/z: 403.1294, found 403.1335 $[M+H]^+$.

Resolution of (±)-saphenic acid, 6-(1-hydroxyethyl)-phenazine-1-carboxylic acid ((±)-3). The racemic acid (±)-**3** (537 mg, 2 mmol, 1 equiv) was dissolved in minimum amount hot MeOH (25 mL). (−)-Brucine (789 mg, 2 mmol, 1 equiv) was added and dissolved immediately. The mixture was allowed to cool to room temperature and left at 4°C for 30 h in a sealed flask containing water in order to allow diffusion between the solvents. The mixture was filtered and the mother liquor was concentrated in vacuo. Both portions of crystals were stirred in NH_4OH (25% solution, 50 mL) for 1 h at ambient temperature. The free brucine was removed by extraction with CH_2Cl_2 (×3). The aqueous phase was acidified by 4 M HCl (aq) to pH 4, extracted with CH_2Cl_2 (×3), dried over $MgSO_4$ and concentrated in vacuo to yield the free acid.

(−)-(*R*)-3 was obtained in 92% yield as yellow crystals from the less soluble salt. Anal. chiral HPLC (254 nm: 83% *R*, 17% *S*) R_T (*R*) = 28.8 min. Mp 204–206 °C. $[\alpha]_D^{22}$ −19.7 (*c*0.13, $CHCl_3$), $[\alpha]_D^{22}$ + 42.0 (*c*0.23, DMSO). Calculations of these specific rotations as well as the following are based on enantiopurity of the sample measured, thus resemble the specific rotation of the pure enantiomer.

(+)-(*S*)-3 was obtained in 70% yield as yellow crystals from the more soluble salt. Anal. chiral HPLC (254 nm: 10% *R*, 90% *S*) R_T (*S*) = 33.0 min. Mp. 203–205 °C. $[\alpha]_D^{22}$ + 20.0 (*c*0.41, $CHCl_3$).

(−)-(*R*)- and (+)-(*S*)-allyl saphenate, (−)-(*R*)- and (+)-(*S*)-6-(1-hydroxy-ethyl)-phenazine-1-carboxylic acid allyl ester (4). Procedure: see racemic (±)-**4**.

(−)-(*R*)-4 was obtained from **(−)-(*R*)-3** in 84% yield as yellow crystals. Anal. chiral HPLC (254 nm: 69% *R*, 31% *S*) R_T (*R*) = 11.2 min. Mp 99–101 °C. $[\alpha]_D^{22}$ −27.3 (*c*0.79, $CHCl_3$).

(+)-(*S*)-4 was obtained from **(+)-(*S*)-3** in 82% yield as yellow crystals. Anal. chiral HPLC (254 nm: 21% *R*, 79% *S*) R_T (*S*) = 12.7 min. Mp 102–104 °C. $[\alpha]_D^{22}$ + 34.3 (*c*0.70, $CHCl_3$).

(−)-(*R*)- and (+)-(*S*)-6-[1-(2-allyloxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid allyl ester (9). Procedure: see racemic (±)-**9**.

(−)-(*R*)-9 was obtained from **(−)-(*R*)-4** and **7** in 40% yield as a yellow oil. 16% (−)-(*R*)-**4** was recovered. Anal. chiral HPLC (254 nm: 81% *R*, 19% *S*) R_T (*R*) = 13.2 min. $[\alpha]_D^{22}$ −53.5 (*c*0.65, $CHCl_3$).

(+)-(*S*)-9 was obtained from **(+)-(*S*)-4** and **7** in 55% yield as a yellow oil. 31% (+)-(*S*)-**4** was recovered. Anal. chiral HPLC (254 nm: 16% *R*, 84% *S*) R_T (*S*) = 14.9 min. $[\alpha]_D^{22}$ + 50.0 (*c*0.63, $CHCl_3$).

Synthesis of (−)-(*R*)- and (+)-(*S*)-6-[1-(2-hydroxy-6-methyl-benzoyloxy)-ethyl]-phenazine-1-carboxylic acid (1). Procedure: see racemic (±)-**1.**

(−)-(*R*)-1 was obtained from **(−)-(*R*)-9** in 88% yield as yellow-green crystals. Mp. 159–164 °C. Anal. chiral HPLC (254 nm: 90% *R*, 10% *S*) R_T(*R*) = 54.2 min. $[\alpha]_D^{22}$ −150.6 (*c* = 0.79, $CHCl_3$).

(+)-(*S*)-1 was obtained from **(+)-(*S*)-9** in 71% yield as yellow-green crystals. Mp 162–165 °C. Anal. chiral HPLC (254 nm: 22% *R*, 78% *S*) R_T (*S*) = 49.2 min. $[\alpha]_D^{22}$ + 176.9 (*c*0.34, $CHCl_3$).

Antimicrobial assay. Minimum inhibitory concentrations (MICs) were estimated using an agar cup assay. Bacterial strains were obtained form the American Type Culture Collection (ATCC) or from the collection of clinical isolates at Leo Pharma A/S. Colonies from fresh overnight cultures were resuspended in saline water to 0.5 MacFarland corresponding to 10^8 CFU/mL. A total of 200 mL Mueller Hinton agar (Oxoid) at 48 °C was inoculated at a concentration of 10^6 CFU/mL and poured into square Petri dishes (245×245 mm). Holes were made in the inoculated plates and 200 μL of each sample was disposed into each hole. Dilution series for Saphenamycin (racemate and enantiomers) contained six dilutions between 0.25 and 125 μg/mL. For *Streptococci*, Mueller Hinton agar was supplemented with 5% sheep blood. Plates were appropriately incubated and zone diameters of growth inhibition were measured using an electronic caliper. MIC values were estimated using linear regression curve between the zone diameter of growth inhibition and the $\log_2$ of the sample concentration. MIC values lower than 0.25 μg/mL were determined by extrapolation.

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