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Dual Dehydrosqualene/Squalene Synthase Inhibitors: Leads for Innate Immune System-Based Therapeutics

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With the rapid rise in bacterial drug resistance,[1] there is great interest in developing innovative approaches to anti-infective therapy. For example, in the United States, more people die from *Staphylococcus aureus* infections than die from human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS).[2] Alternative approaches are thus of interest, and as discussed in a recent National Research Council (US) report,[1b] these include targeting virulence factors, as well as boosting innate immunity. In recent work, we showed that blocking formation of the carotenoid virulence factor staphyloxanthin with BPH-652 (**1**, Figure 1) rendered staph bacteria noninfective and susceptible to immune system clearance mediated by reactive oxygen species,[3] and in other work, we showed that inhibiting squalene biosynthesis in neutrophils with **2** generated antibacterial, neutrophil extracellular traps (NETs).[4] The targets, dehydrosqualene synthase (CrtM, for **1**) and squalene synthase (SQS, for **2**), are both involved in the first committed steps in carotenoid and sterol biosynthesis, the conversion of two farnesyl diphosphate molecules to form presqualene diphosphate (Figure 2a). These findings led us to contemplate that it might be possible to develop dual activity CrtM/SQS inhibitors that simultaneously block formation of the *S. aureus* virulence factor staphyloxanthin—removing the golden protective shell of the bacterium (Figure 2b)—while at the same time stimulating host antimicrobial NET formation (Figure 2c). This would represent a novel, dual-targeting approach to antibacterial therapy. Here, we report the discovery of such lead compounds.

First, we carried out an in silico high-throughput screen (HTS) for new CrtM inhibitor leads, since current inhibitors such as compound **1** are poor NET inducers (Table 1). Likewise, the use of statins to block pigment formation is not feasible since the K_i value for inhibition of *S. aureus* HMG-CoA reductase by statins is approximately 10^4 larger than for inhibition of

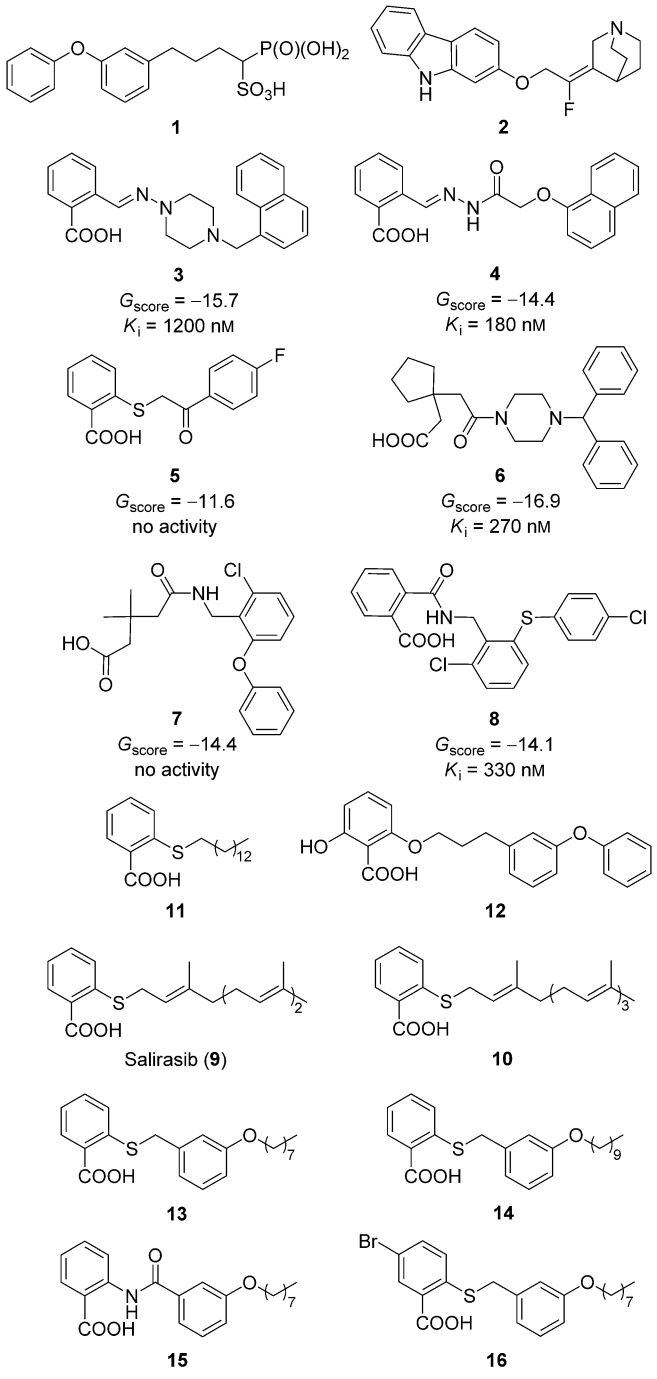

Figure 1. Structures of **1**–**16**. Hits **3**–**5** were identified from the Life Chemicals Inc. library, while hits **6**–**8** were found in the Maybridge Chemical Co. library. The Glide G_{score} and K_i values against dehydrosqualene synthase (CrtM) are given for these screening hits.

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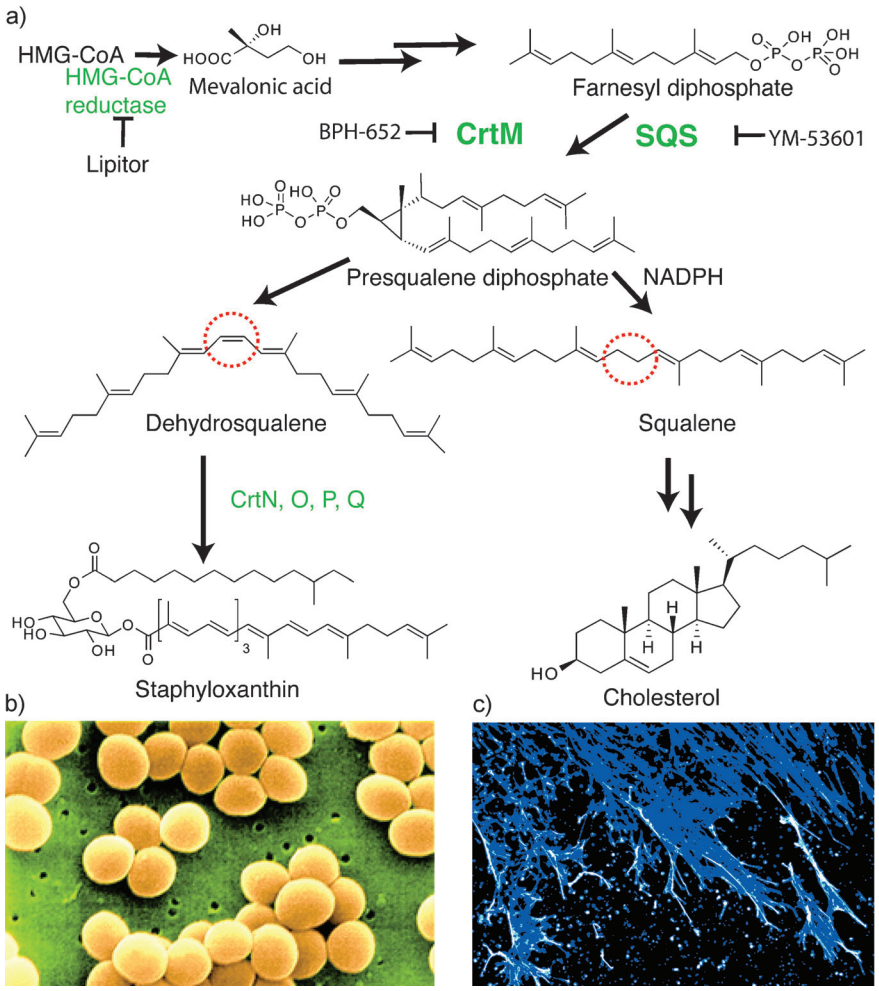

Figure 2. Head-to-head prenyltransferase drug targets. a) Schematic illustration of the reactions catalyzed by two head-to-head prenyl transferases: dehydrosqualene synthase (CrtM) and squalene synthase (SQS). The end products are the carotenoid, staphyloxanthin or the sterol, cholesterol. b) Staphyloxanthin gives *S. aureus* its (eponymous) gold coloration and is a major virulence factor. c) Blocking cholesterol biosynthesis in neutrophils at the level of SQS results in the formation of neutrophil extracellular traps (NETs), which have potent antibacterial activity.

host HMG-CoA reductase, due to large class I/class II enzyme structural differences.[5] We used the CrtM–**1** structure reported previously (PDB ID: 2ZCQ),[3] removing the phosphonosulfonate ligand, while retaining the two Mg^{2+} ions and water molecules within 5 Å around **1**. We used the virtual screening workflow in Glide,[6] and screened 2372 compounds from a commercially available antibacterial library (Life Chemicals Inc., Burlington, Canada; http://www.lifechemicals.com) and 14400 compounds from the HitFinder library (Maybridge Chemical Co., Trevillett, UK; http://www.maybridge.com), identifying hits **3**–**5** from the first library and **6**–**8** from the second library (Figure 1). Each of these compounds has a carboxylic acid attached either to a benzene ring or another hydrophobic group, plus, in each case there is a more distal, aromatic feature. We attempted crystallization of **3**–**8** with CrtM and obtained crystals that diffracted well for **6** (2.07 Å) and **8** (2.00 Å). Full crystallographic data and structure refinement details are given in Table S1 and the Methods section in the Supporting Information. Ligand electron densities are given in Figure S1 a and b of the Supporting Information.

The structures of **6** and **8** bound to CrtM are shown in Figure 3. In both cases, what can immediately be seen is that the more distal, hydrophobic, diphenylmethane (**6**) or diphenylsulfide (**8**) side chains bind to both the S1 as well as the S2 hydrophobic pockets[7] in CrtM that are involved in farnesyl diphosphate (FPP) substrate binding. Furthermore, in both cases, one phenyl group is centered at ~C10 in the S2 *S*-thiolo-farnesyl diphosphate (FSPP), an FPP-like inhibitor, side chain position, while the second phenyl group overlaps the position occupied by the chain terminus of FSPP in S1. However, with hit compound **6**, the carboxyl group has electrostatic interactions with R171 and R265 in the polar group region in S2 (Figure 3 b), while in the crystal structure of CrtM–**8**, interactions are with the carboxyl and Mg_A^{2+} in S1 (Figure 3 d). Both inhibitors have good activity against CrtM (~300 nM) and high IC_{50} values (>20 μM) against three human cell lines (Table 1).[3] However, these initial screening hits were not active in an *S. aureus* pigment inhibition assay (IC_{50} >100 μM).

Based on the preponderance of *ortho*-substituted benzoic acids in the HTS hits, i.e., three out of four compounds having, on average, a K_i value of ~400 nM against CrtM, we next carried out a similarity search (based on **3**–**8**) in the PubChem database[8] to find other leads. An interesting result was that the *S*-thiolo-farnesyl benzoic acid, Salirasib (**9**), was returned as a similar compound. Salirasib blocks Ras signaling,[9] and it is currently in clinical trials for the treatment of various cancers, and since it is orally available, it appeared an interesting potential lead. Salirasib (**9**) had a K_i value of 110 nM in the CrtM enzyme assay but the *S. aureus* pigment inhibition was again poor (IC_{50} = 75 μM; Table 1). We reasoned that a longer chain (geranylgeranyl) analogue, as in compound **10**, might have better enzyme inhibition activity, since it is known that geranylgeranyl diphosphate can bind to the S2 site of CrtM.[7] Indeed, compound **10** had a K_i value of 28 nM against CrtM, and an IC_{50} value of 4 μM in the pigment inhibition assay.

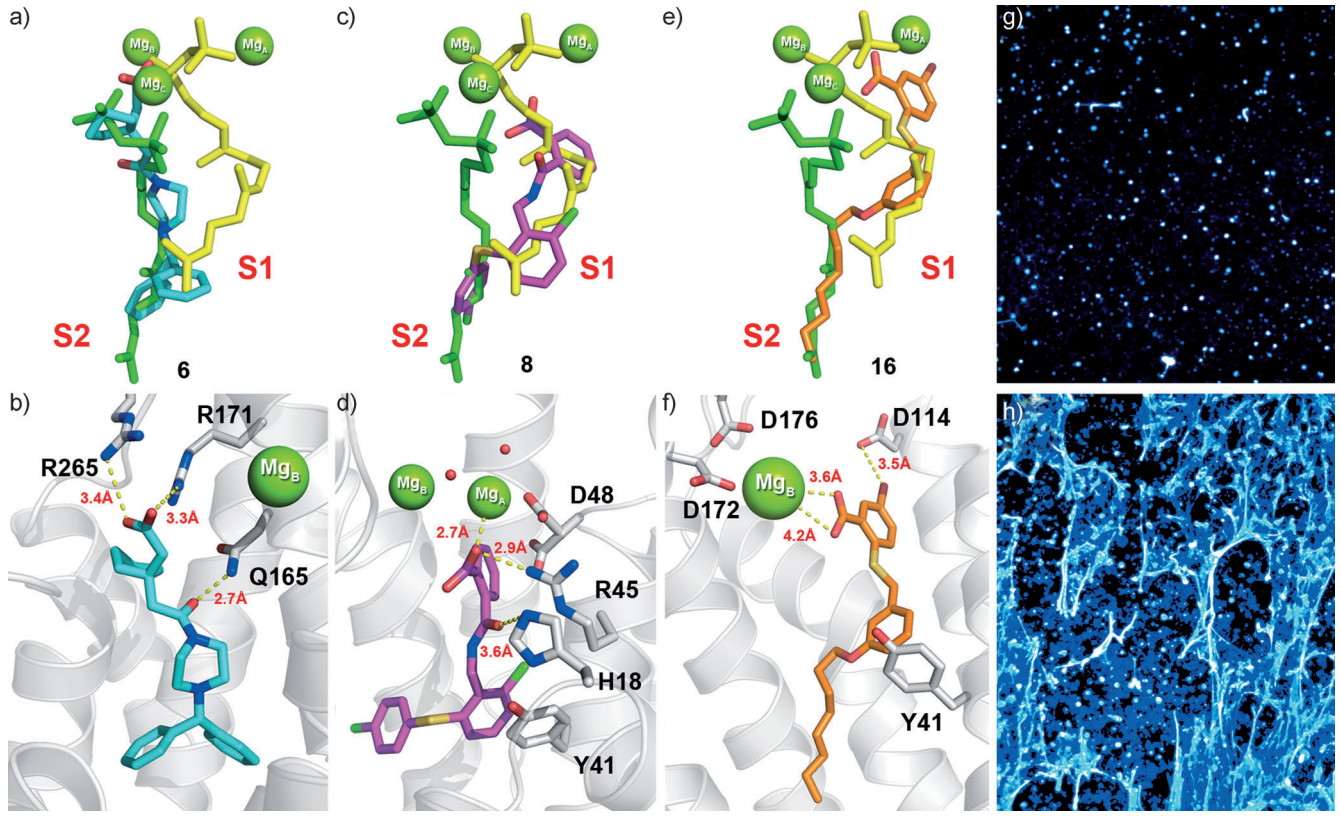

Figure 3. Crystal structures and neutrophil extracellular traps (NETs) formation. a) Superimposition of compound **6** (cyan, PDB ID: 3TFN) on two FSPP molecules (green and yellow; PDB ID: 2ZCP). b) Polar CrtM residues interacting with compound **6**. c) Superimposition of compound **8** (pink, PDB ID: 3TFP) with FSPP (PDB ID: 2ZCP). d) Mg_A^{2+} and CrtM residues interacting with compound **8**. e) Superimposition of compound **16** (orange, PDB ID: 3TFV) with FSPP (PDB ID: 2ZCP). f) Mg_B^{2+} and CrtM residues interacting with compound **16**. g) Phosphate-buffered saline (PBS) control with phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils. h) Extensive sheets of NETs are formed on activation of neutrophils with compound **14** at a concentration of 5 μM.

We then produced five analogues (**11**–**15**) based on compound **10**. Among them, derivative **14** had a K_i value of 120 nM against CrtM and an IC_{50} value of 120 nM in the pigment inhibition assay. We attempted to crystallize each compound **9**–**15** bound to CrtM using both soaking and co-crystallization techniques, but found no complete ligand densities. Soaking **14** yielded crystals that diffracted to 2 Å, but there was missing density in the benzoate region due to structural disorder. Thus, we made bromo-analogue **16**, and one crystal diffracted to 3.00 Å (Table S1 in the Supporting Information). Although this is only moderate resolution, the electron density (Figure S1 c in the Supporting Information) clearly indicates that the long alkyl chain binds to the S2 site (in orange, Figure 3 e) and is essentially superimposed on the S2 FSPP side chain (in green, Figure 3 e), while the benzyl group forms a T-shaped π-stacking interaction with Y41, and the bromobenzoate fragment binds in the S1 site, interacting with Mg_B^{2+} (Figure 3 f).

Table 1. Enzyme, pigment, cell-growth inhibition and neutrophil extracellular trap (NET) induction results.

Compd	K_i [μM] hSQS	K_i [μM] CrtM	PI[a] [μM]	CA[b] [μM]	NET formation[c]	TI[d]
1	0.37	0.09	0.12	>200	–	~0
4	0.78	0.18	>100	32	ND	<0.3
6	0.53	0.27	>100	15.7	ND	<0.2
8	9	0.34	100	101	ND	<1
9[e]	0.37	0.11	75	15.8	+	0.2
10	0.3	0.028	4	15.6	+	3.9
11	0.06	0.052	0.92	22	–	24
12	2	0.028	9.0	29	–	39
13	1.25	0.16	0.15	13.3	+	89
14	0.2	0.12	0.12	16	+++	133
15	0.3	0.09	ND	10.4	+	ND
16	0.2	0.08	ND	37	++	ND

[a] ND indicates not determined since there was bacterial cell-growth inhibition, and so pigment inhibition (PI) could not be accurately measured. [b] Cancer average (CA) is the average activity against the MCF-7, NCI-H460 and SF-268 cell lines as described in Ref. [3]. [c] NET formation was not determined (ND) if there was no pigment inhibition. [d] Therapeutic index (TI) represents the ratio: IC_{50} (human cell)/IC_{50} (pigment) and was not determined if there was bacterial growth inhibition. [e] Salirasib (**9**).

We tested compounds **9**–**16** for human cell-growth inhibition in order to see whether or not these compounds are highly toxic. By determining the therapeutic index (TI), we found that the most potent leads (**13**–**14**) also had the best TI value of approximately 100 (Table 1). We then screened the small library of compounds synthesized for SQS inhibition. The most active compound that also blocked pigment formation and had a good TI value was **14**, which has a K_i value of approximately 200 nM against SQS (Table 1). More importantly, at

a concentration of 5 μM, compound **14** caused extensive NET formation in phorbol 12-myristate 13-acetate (PMA)-stimulated neutrophils,[4] as evidenced by staining with monoclonal antibodies against neutrophil myeloperoxidase (MPO) (Figure 3 g and h).[4] The potent CrtM/SQS inhibitor **16** was also active in NET formation (Table 1), but unlike **14**, it also inhibited *S. aureus* growth. This is similar to the observation that other *meta*-substituted benzoic acids inhibit *S. aureus* growth,[10] suggesting that direct antibacterial activity, virulence factor inhibition, as well as innate immune system activation via NET formation, might all be targeted by a single compound. Taken together, these results suggest new avenues for the treatment of *S. aureus* infections in the current era of increasing resistance to conventional antibiotic therapy.

Acknowledgements

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Keywords: immunology · isoprenoid biosynthesis · neutrophil extracellular traps · virulence factor · X-ray diffraction

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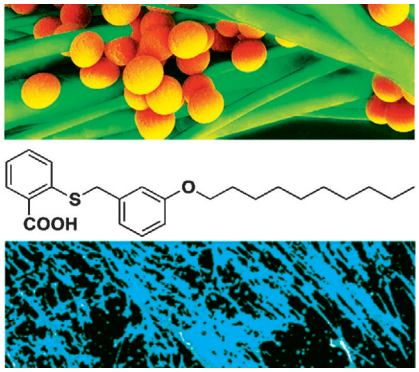
Double whammy! Small molecules that inhibit *Staphylococcus aureus* dehydrosqualene synthase (CrtM) or host squalene synthase (SQS) are of interest as novel, innate immunity-based therapeutics, blocking virulence or stimulating antibacterial neutrophil extracellular trap (NET) formation. The discovery of leads that do both represents a new route to treating staph infections.



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