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Preparation of novel anthranilic acids as antibacterial agents: Extensive evaluation of structural and physical properties on antibacterial activity and human serum albumin affinity

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Abstract—In the past few years a significant effort has been devoted by Pharmacia toward the discovery of novel antibiotics. We have recently described the identification of an anthranilic acid lead **1** and the optimization resulting in the advanced lead **2**. In this report, we describe the preparation of several selected analogs to probe the dependency of this template for antibacterial activity and the affinity these compounds have for human serum albumin (HSA). These analogs illustrate that decreased affinity for HSA can be achieved while retaining relevant antibacterial activity. The most important factor for reduced HSA affinity is decrease in log *P* rather than a structural change.

Emergence of bacterial resistance is a significant problem in the treatment of bacterial infections,[1] and this has fueled a continual search for novel antibiotics resulting in numerous commercially available products. We recently reported[2] on the optimization of acid **1** resulting in the discovery of lead compound **2** which displayed potent broad-spectrum antibacterial activity (Fig. 1). Extensive results from biological assays indicated that high affinity to human serum albumin[3] (HSA) was the main reason for lack of in vivo activity of **2**. An observation in the literature describes the utility of anthranilic acids as high affinity ligands for HSA.[4] There are numerous ways to evaluate the affinity of different ligands for HSA such as chromatographic, spectroscopic, and displacement of a high affinity ligand from HSA.[5] A very common approach described in the literature is the addition of serum to the biological assay. The difference of activity in the presence and absence of serum can be related to HSA affinity.[6] The affinity to HSA can be described as binding constant (K_d), % free fraction, or a ratio of activity in the presence or absence of serum.

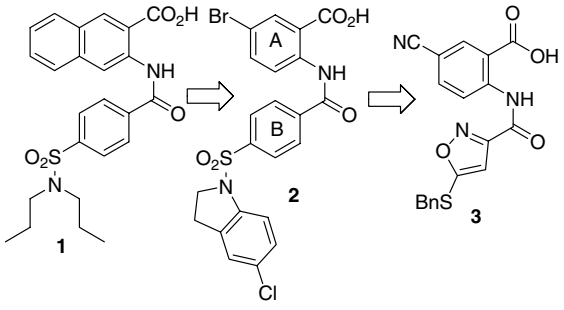

Figure 1. Progression in lead optimization.

The difficulty inherent in protein binding can be illustrated by looking at the case of a compound with a K_d of 1 μM for HSA at a fixed compound concentration. At the biologically relevant concentration of 600 μM HSA, the free fraction will only be 0.2%. A decrease in affinity for HSA such as an increase in the K_d to 10 μM results in a free fraction of 1.6%. An early example of the evaluation of antibiotics' affinity to HSA was the measurement of MIC activity of a range of commercially available antibiotics in the absence and presence of

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100% serum.[7] Highly bound drugs such as Dicloxacillin require good pharmacokinetic properties and significantly more potency to exert the same effect as less protein bound drugs.[8] In general, it is only the free fraction of a drug that is of biological consequence (or relevance?). A rule of thumb has been proposed that in order to observe in vivo activity with 80% certainty, $C_{max}^{free}/MIC \geqslant 8:1$.[8b] We elected to utilize the primary MIC assay in the presence and absence of serum as a primary indicator for monitoring improvements in potency and decreases in HSA affinity. The major advantage of the MIC assay was high throughput, acceptable accuracy, and a measurement of overall HSA affinity. Utilizing this approach we recently reported on optimization of **2** targeting the reduction of its affinity for human serum albumin (HSA) which resulted in advanced compound **3**.[9] A detailed description of the SAR with regard to structural tolerance correlation with lipophilicity and how that relates to HSA affinity are presented in this report.

The potential for rational design of new anthranilic analogs with decreased HSA affinity. A rational approach is the utilization of protein crystallography to engineer in non-favorable interaction. A few structures of HSA have been determined[10] and a recent report for a successful rational design of non-favorable interaction has been described for Diflunisal analogs.[11] In order to evaluate if this would be a feasible approach here we elected to prepare analogs that were confirmationally rigid, displaying a bulky group where SAR indicated that substitution was allowed. This could be accomplished on each periphery of the core such as example **4** (Fig. 2).

The analogs were prepared by sequential amide coupling followed by a Suzuki reaction and finally a TFA deprotection in modest overall yield, Schemes 1 and 2. This approach allowed us to rapidly prepare numerous analogs in each series (data not shown). The key observation is that compounds **6** and **8** were active as

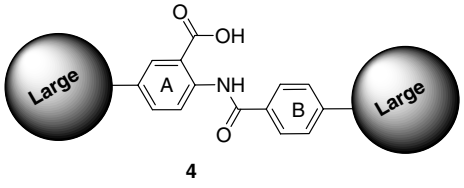
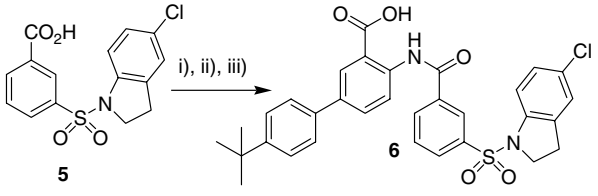
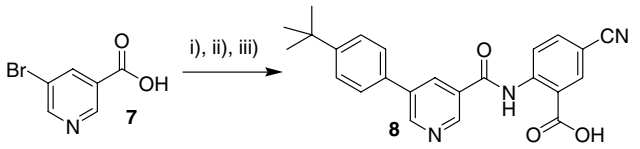


Figure 2. Design of probe analogs.



Scheme 1. Reagents: (i) $(COCl)_2$, DCM, pyridine, *t*-butyl-2-amino-4-iodo-benzoate (55%); (ii) 4-*t*-butyl-benzene boronic acid, tetrakis(triphenylphosphine) palladium, Na_2CO_3, THF/water (61%); (iii) TFA (75%).



Scheme 2. Reagents: (i) $(COCl)_2$, DCM, pyridine, *t*-butyl-2-amino-4-cyano-benzoate (61%); (ii) 4-*t*-butyl-benzene boronic acid, tetrakis(triphenylphosphine) palladium, Na_2CO_3, THF/water (75%); (iii) TFA (57%).

Table 1. Antibacterial activity of new probe analogs

Compound	MIC[a] (μg/mL)		
	SAUR[b]	SAUR[c]	SAUR[d]
6	16	>128	>128
8	32	>128	>128

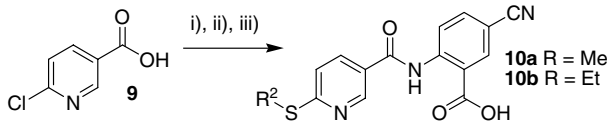
[a] Minimal inhibitory concentration.
[b] *Staphylococcus aureus* UC 9218.
[c] *Staphylococcus aureus* UC 9218 + 5% serum. Human serum (male, from Sigma) was thawed at room temperature, then placed in a 56 °C water bath for 30 min. The serum was then filtered using a 0.2 μm filtration system.
[d] *Staphylococcus aureus* UC 9218 + 10% serum.

antibacterial agents (Table 1). Despite our efforts to bump these analogs out of their HSA binding site via their large linear size, they were still excellent substrates for HSA. These observations diminished our hope that rational design via steric interaction with HSA would be a viable path forward in new analog design. This was not surprising since HSA binding characterization of the deschloro analog of **2** was found to have high affinity to both the ibuprofen ($K_d \sim 1$ μM) and the warfarin site ($K_d < 1$ μM).[12] Because of the high affinity for multiple binding sites (vide supra), structural alteration for affinity for one site could be tolerated by the other and vice versa.[13] The net result is still high overall affinity for HSA.

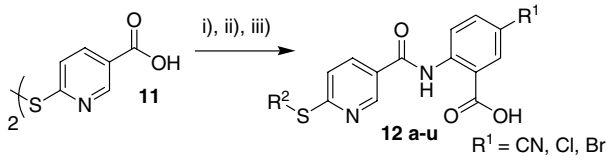
Systematic evaluation of physical properties with antibacterial activity and HSA affinity. A less direct approach to reducing protein binding is to guide SAR based on the correlation of physical parameters with bioactivity and HSA affinity. This has been a very common approach since the initial disclosure by Hansch applied to a series of penicillins.[14] There are numerous reports on the correlation of physical parameters with activity of antibacterial agents mainly for penicillins, and in general, the major correlation is between lipophilicity and HSA affinity.[15] In addition, acidic groups are known to increase affinity for HSA. A recent publication describes a detailed analysis where the major factor for HSA affinity appears to be the compound's lipophilicity.[16] In addition to lipophilicity, the analysis indicated that cyclic structures especially six-membered rings have high affinity for HSA. Affinity was decreased by branching or conversion to acyclic systems.

In order to evaluate these possibilities, we selected to prepare a series of pyridyl-thioethers, where we could modulate both the log *P* as well as the nature of the substituent in a systematic fashion. Our initial approach to

the synthesis of these compounds was to prepare the intact extended analog and displace a chloride with a thiolate in the last step. This worked very well for methyl and ethyl thiolates, Scheme 3. However, when the chain length was increased to propyl none of the desired material was obtained despite several optimization attempts. An alternative approach was designed that relied on the reduction of disulfide followed by alkylation of the derived anion with an alkyl halide, Scheme 4. The acid was then coupled to the amine. In this instance, acid chloride formation required the harsher conditions of thionyl chloride in order to provide a reliable yield of the desired amide. This alternative approach allowed us to prepare a significant number of structurally diverse thioether analogs.



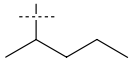
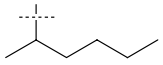
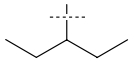
Scheme 3. Reagents: (i) $(COCl)_2$, DCM, pyridine, *t*-butyl-2-amino-4-cyano-benzoate (55%); (ii) Sodium thiolate, dioxane, reflux, (R = Me 86%, R = Et 59%); (iii) TFA (70–90%).



Scheme 4. Reagents: (i) $NaBH_4$, alkyl halide (25–100%); (ii) $SOCl_2$, DCM, pyridine *t*-butyl-2-amino-4-bromo (or chloro, or cyano)-benzoate (17–74%); (iii) TFA (52–92%).

In general, the thioether analogs are very active against *Staphylococcus aureus* with numerous analogs having MIC of 0.125 μg/mL, Table 2. Numerous analogs are very potent in the presence of added serum in the *S. aureus* MIC assay. In most instances, as the performance in presence of serum improves the spectrum of activity deteriorates (data not shown). There is not a linear correlation between decrease in *C*log *P* and improved performance in the presence of serum. That is to some extent contradictory to numerous observations in the literature.[15,16] Rather, it appears that improvement in potency with increase in *C*log *P* is greater than increase in

Table 2. Antibacterial activity of selected pyridyl thioether analogs in the presence and absence of serum

R^2	Compound	R^1	MIC SAUR 9218 (μg/mL)		*R* (10%)[a]
			0% serum	10% serum	
Me	**10a**	CN	4	32	8
Et	**10b**	CN	0.5	8	16
n-Pr	**12a**	CN	0.5	32	64
n-Bu	**12b**	CN	0.25	16	64
n-C_5H_{11}	**12c**	CN	0.125	4	32
n-C_6H_{13}	**12d**	CN	0.125	8	64
n-C_6H_{13}	**12e**	Br	0.125	64	512
n-C_6H_{13}	**12f**	Cl	0.125	64	512
n-C_9H_{19}	**12g**	CN	1	>128	>128
n-$C_3H_6OC_2H_4OCH_3$	**12h**	CN	1	4	4
i-Pr	**12i**	CN	1	16	16
i-Pr	**12k**	Br	1	32	32
i-Bu	**12l**	CN	0.5	8	16
	**12m**	CN	0.125	16	128
	**12n**	CN	0.125	16	128
	**12o**	CN	0.25	8	32
	**12p**	CN	0.5	32	64
	**12r**	Br	1	64	64
	**12s**	CN	0.5	16	32
	**12t**	CN	0.125	4	32
	**12u**	Br	1	32	32

[a] *R* = [(MIC 10% serum)/(MIC 0% serum)].

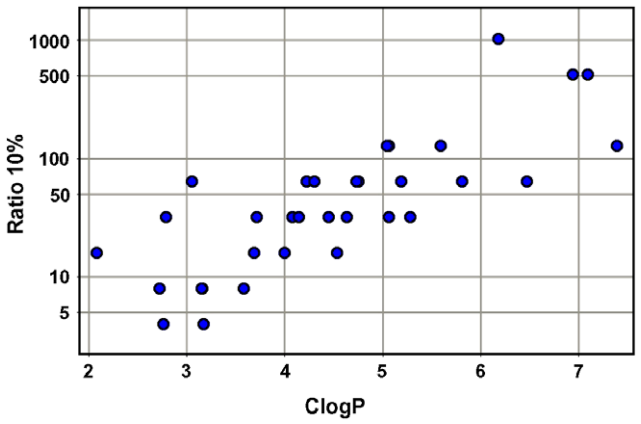


Figure 3. Plot of *C*log *P* of prepared analogs vs. ratio (ratio defined as MIC 10% serum/MIC 0% serum).

HSA affinity. This trend is well exemplified by the linear series C_1–C_9 (**10a**–**12g**), where it appears that a C5 tether is optimal in **12c**. It is difficult to compare the acyclic series with the cyclic series (**12p**–**u**), but in general it appears that the cyclic series does not perform as well. A more interesting observation is that branched acyclic compounds appear to be performing the best in both the C_3 tether (**12a** vs. **12i**) and the C_4 tether (**12b** vs. **12l**). The branched analog is more potent and performs better in the presence of serum as judged by MIC or the *R*-value (ratio). Therefore, analogs **12o**, **12n**, and **12m** were prepared. Their disappointing performance demonstrates the difficulty in analyzing and predicting biological activity in the presence of serum. The addition of oxygen atoms in the tether in general improves the performance in the presence of serum.

A more detailed analysis of HSA affinity was performed on **12h** indicating an affinity (K_d) for the ibuprofen site was 14 μM and an overall HSA affinity was ~0.7 μM. This is a significant reduction compared to compound **2** especially at the ibuprofen site. In general for this series it appears that performance in the presence of serum is difficult to relate to a single parameter such as *C*log *P*, rather it is a complex correlation of increased potency, *C*log *P*, and branching that is dictating performance. In general, increased *C*log *P* confers more potent compounds with a break point of 6–9 carbons in the thioether substituent where HSA affinity dominates potency improvement. In general lower-branched thioethers have the best ratio of MIC activity, the cyclic ones are the worst, and linear ones sit in the middle. Our observations correlate well with the recent publication on protein binding describing relationships of *C*log *P*, branching, and cyclic parameters.[16] The most generic correlation of lipophilicity and protein binding is depicted in Figure 3. The *R* factor is a measurement of protein binding and it is obvious that there is an excellent correlation of *C*log *P* with *R*. There was not observed any correlation with other physical parameters such as topical polar surface area or MW.

Conclusion. In this paper, we described some of the key observations we made regarding antibacterial SAR of this novel template and the properties influencing its affinity for HSA. Based on the synthesis of compounds **6** and **8**, we believe that rational design is unlikely to guide the design of less protein bound analogs. On the other hand, the close correlation of *C*log *P* with HSA affinity provides a strategy for designing new analogs. These compounds bind to multiple sites on HSA and the *C*log *P* modification has dramatic effect on affinity of the compounds for the ibuprofen site. The overall effect on decreased HSA affinity is more modest. The future focus is on improving potency in new compounds while retaining a low *C*log *P*. Potency enhancements which are the results of *C*log *P* increases are unlikely to provide analogs with the desired properties.

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