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Salicylanilide esters of *N*-protected amino acids as novel antimicrobial agents

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ABSTRACT

A series of novel, highly antimicrobial salicylanilide esters of *N*-protected amino acids were synthesized and characterized. Their in vitro antimicrobial activity against eight fungal strains and *Mycobacterium tuberculosis* was determined. The compounds had the highest level of activity against *Aspergillus fumigatus*, *Absidia corymbifera* and *Trichophyton mentagrophytes*, and these levels were higher than that of the standard drug fluconazole. In addition, three compounds showed interesting antituberculosis activity, with inhibition ranging from 89% to 99%. (*S*)-4-Chloro-2-(4-trifluoromethylphenylcarbamoyl)-phenyl 2-benzyloxy-carbonylamino-propionate had the highest level of both antifungal and antimycobacterial activity. The structure–activity relationships of the new compounds are discussed.

Salicylanilides (2-hydroxy-*N*-phenylbenzamides) have been reported as a class of compounds with a wide variety of interesting biological activities, including antimycobacterial and antifungal effects.[1–5] Although the antibacterial activity involves multiple mechanisms, these compounds have been shown to be inhibitors of the two-component regulatory systems (TCS) of bacteria.[6,7] The most recent studies identified them as selective inhibitors of interleukin-12p40 production[8] and inhibitors of the protein kinase epidermal growth factor receptor (EGFR PTK).[9,10]

A dramatic rise in the incidence of life-threatening systemic fungal infections has been observed over the past two decades.[11] This can be ascribed to an increase in the number of immuno-compromised patients due to the growth of the number of HIV-infected individuals, cases of cancer chemotherapy and the indiscriminate use of antibiotics. The majority of antifungal agents in clinical use suffer from various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Hence, there is a great demand for new antifungal agents belonging to a wide range of structural classes, acting selectively on novel targets and with fewer unwanted side-effects.[12]

No new drug class has been introduced in the past 50 years for the treatment of tuberculosis (TB).[13] Currently, patients require 6–9 months of treatment. This long period leads to the lack of compliance, which in turn can be responsible for the relapse and incidence of MDR-TB strains.[14] There is an urgent need to develop a new potent and fast-acting antituberculosis drug with a low toxicity profile. The new drug must be suitable for use in conjunction with the drugs currently used for the treatment of HIV infection, which are active against both growing and latent infections of TB.

There is a need for antibacterial agents with a novel mechanism of action and the salicylanilides are promising candidates. An electron-withdrawing group on the salicyloyl ring and hydrophobic groups on the anilide moiety, as well as the 2-hydroxy group, are essential for their optimal antimicrobial effect. The salicylanilides substituted with halogens in both parts meet the requirements and form the most active derivatives possessing antifungal and antituberculosis activity against some atypical strains of mycobacteria.[1]

Their unsuitable physical properties led us to modify the structure by esterification of amino acids with suitable salicylanilides. In addition, amino acids facilitate the possibility of targeting drugs as a drug delivery system. Prepared esters can be considered as prodrug forms of salicylanilides with better bioavailability and, due to the higher degree of liphophilicity, more efficient transport through the mycobacterial cell membrane.

Here, we report the synthesis, experimental determination of liphophilicity by reversed phase high performance liquid chromatography (RP-HPLC), antifungal and antimycobacterial activity, as well as SAR of a series of salicylanilide derivatives.

The starting salicylanilide compounds were obtained by the reaction of 5-chlorosalicylic acid and appropriate anilines in a microwave reactor in the presence of PCl_3 and chlorobenzene as a solvent. A method using dicyclohexycarbodiimide (DCC) in anhydrous DMF was chosen for esterification of the *N*-benzyloxycarbonyl amino acids (Scheme 1).[15]

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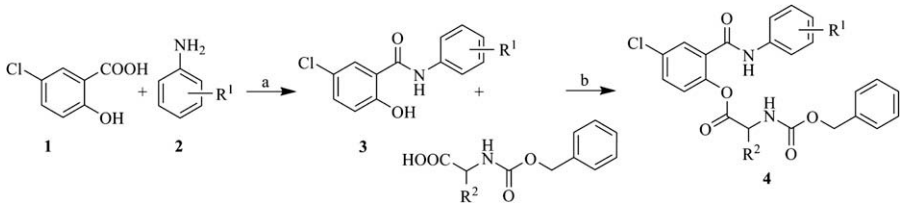

Scheme 1. Synthesis of *N*-protected amino acid esters. Reagents: (a) PCl_3, chlorobenzene, microwave reactor; (b) DCC, DMF.

All the substituted salicylanilide esters of amino acids were analyzed for lipophilicity using RP-HPLC. The procedure was performed under isocratic conditions with methanol as an organic modifier in the mobile phase using an end-capped non-polar C_{18} stationary RP column.

The hydrophobicity (log*P*/Clog*P* data) of each of the compounds **4a**–**4j** were calculated using two commercially available programs (CS ChemOffice 9.0 and ACD/Log*P* 1.0) and measured by RP-HPLC determination of the capacity factor *k* with the subsequent calculation of log *k*. The results are shown in Table 1(A).

Log*k* data specify the lipophilicity within this series of compounds. Neither of the computer programs resolved lipophilicity parameters within the series of enantiomers; nevertheless, the chiral compounds **4b**, **4c**, **4e**–**4g** and **4i** were measured several times with the same results. The differences in log*k* parameters for individual *R*/*S*-enantiomers cannot be explained on the basis of the results presented here.

As expected, the lipophilicity of compounds **4a–j** substituted in the R^2 position increased in the order: R^2 = H (compounds **4a**, **4d**, **4h** and **4j**) < R^2 = CH_3 (compounds **4b**/**4c** and **4i**) < R^2 = $CH(CH_3)_2$ (compounds **4e**/**4f**) < R^2 = $CH_2C_6H_5$ (compound **4g**). The dependence between log*k* and the alkyl/phenylalkyl substituents in the individual series of the compounds (R^2 = H, $-CH_3$, $-CH(CH_3)_2$, $-CH_2C_6H_5$) is approximately linear, as shown in Figure 1.

All the *N*-benzyloxycarbonyl amino acid esters of salicylanilides were tested for their in vitro antifungal and antituberculosis activity.

The in vitro antifungal screening was used for the eight strains at concentrations in the range 62.5–1.95 μmol/L.

Table 1
Lipophilicity and antimicrobial evaluation of the new compounds

Compound	R^1	R^2	log*k*	log*P*/Clog*P* (ChemOffice)	log*P* (ACD/log*P*)
(A) Comparison of the logk values with the calculated lipophilicity of the new compounds					
4a	3-Cl	H	0.6119	4.71/5.14625	5.04 ± 0.48
4b	3-Cl	(*S*)-CH_3	0.6995	5.20/5.45525	5.39 ± 0.48
4c	3-Cl	(*R*)-CH_3	0.6990	5.20/5.45525	5.39 ± 0.48
4d	4-Cl	H	0.6367	4.71/5.14625	5.00 ± 0.47
4e	4-Cl	(*S*)-$CH(CH_3)_2$	0.9421	6.09/6.38325	6.23 ± 0.48
4f	4-Cl	(*R*)-$CH(CH_3)_2$	0.9417	6.09/6.38325	6.23 ± 0.48
4g	4-Cl	(*S*)-$CH_2C_6H_5$	1.0971	6.88/6.87325	7.28 ± 0.40
4h	4-CF_3	H	0.7292	5.07/5.50945	5.03 ± 0.51
4i	4-CF_3	(*S*)-CH_3	0.8149	5.57/5.81845	7.28 ± 0.40
4j	3,4-Cl	H	0.8639	5.27/5.7738	5.91 ± 0.50

	MIC/IC_{80} (μmol/L)								M. tbc H_{37}Rv	
	CA 24h 48h	CT 24h 48h	CK 24h 48h	CG 24h 48h	TB 24h 48h	AF 24h 48h	AC 24h 48h	TM 72h 120h	MIC (μg/mL)	Inh. (%)
(B) The in vitro antifungal and antituberculotic activity of N-benzyloxycarbonyl amino acids esters of salicylanilides										
4a	>62.5	>62.5	62.5	62.5	>62.5	>62.5	62.5	31.25	>6.25	7
	>62.5	>62.5	62.5	>62.5	>62.5	>62.5	62.5	31.25		
4b	31.25	>62.5	31.25	31.25	>62.5	62.5	15.63	7.81	3.13	97
	>62.5	>62.5	31.25	>62.5	>62.5	>62.5	15.63	7.81		
4c	62.5	62.5	31.25	62.5	>62.5	>62.5	31.25	7.81	>6.25	36
	>62.5	>62.5	31.25	62.5	>62.5	>62.5	31.25	7.81		
4d	62.5	31.25	31.25	62.5	62.5	>62.5	31.25	15.63	>6.25	16
	62.5	31.25	31.25	62.5	>62.5	>62.5	62.5	15.63		
4e	>62.5	>62.5	31.25	>62.5	>62.5	>62.5	>62.5	15.63	>6.25	38
	>62.5	>62.5	15.63	>62.5	31.25	>62.5	>62.5	15.63		
4f	>62.5	>62.5	31.25	>62.5	>62.5	>62.5	>62.5	15.63	>6.25	77
	>62.5	>62.5	31.25	>62.5	>62.5	>62.5	>62.5	15.63		
4g	62.5	31.25	15.63	31.25	31.25	>62.5	15.63	7.81	>6.25	16
	62.5	31.25	15.63	31.25	62.5	>62.5	15.63	7.81		
4h	62.5	125	31.25	31.25	31.25	125	31.25	15.63	>6.25	89
	62.5	>125	31.25	>125	31.25	>125	31.25	15.63		
4i	62.5	500	7.81	125	62.5	7.81	31.25	1.95	3.13	99
	250	500	31.25	250	250	15.62	62.5	1.95		
4j	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	62.5	31.25	>6.25	0
	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	31.25		
FLU	0.06	0.12	3.91	0.98	0.24	>125	>125	1.95	—	—
	0.12	>125	15.62	3.91	0.48	>125	>125	3.91		
INH	—	—	—	—	—	—	—	—	0.025–0.2[16]	—

CA, *Candida albicans* ATCC 44859; CT, *Candida tropicalis* 156; CK, *Candida krusei* E28; CG, *Candida glabrata* 20/I; TB, *Trichosporon beigelii* 1188; AF, *Aspergillus fumigatus* 231; AC, *Absidia corymbifera* 272 and TM, *Trichophyton mentagrophytes* 445.

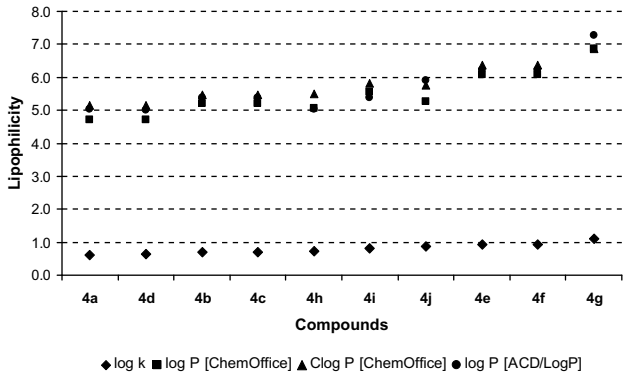

Figure 1. Comparison of log*P*/Clog*P* data calculated using the two computer programs with the experimentally determined log*k* values. The compounds are ordered according to the increase in log*k* values in the series of compounds **4a**–**4j**.

All the esters had the highest level of activity against *Trichophyton mentagrophytes* 445. Most had a higher level of activity than fluconazole, especially against *Aspergillus fumigatus* 231 and *Absidia corymbifera* 272. For the results of the antifungal assays, see Table 1(B).

Compounds **4i**, **4g** and **4b** had the highest level of activity. On the basis of this fact (dependence of antifungal activity/lipophilicity) it can be supposed that hydrophobicity is only a secondary parameter, see Table 1. Compounds **4a** and **4j** had considerably different degrees of lipophilicity but similar levels of activity. The same was observed for compounds **4d**, **4h**, **4e**/**4f** and **4c**/**4b**, **4g**, where the effect of various substituents, including CF_3, Cl and/or H, CH_3, $CH(CH_3)_2$, $CH_2C_6H_5$, with different degrees of hydrophobicity on antifungal activity was minor.

When the position and the identity of individual substitutions were compared, substitution at the $C_{(4)'}$ position seemed to be more important to increase the antifungal activity than substitution at the $C_{(3)'}$ position of the anilide ring. R^2-substitution by a methyl or a benzyl moiety seemed to be better than that by an isopropyl moiety. The benzyl derivative **4g** may be considered as a phenyl cyclic analogue of compound **4i** with a similar level of activity or a benzyl analogue of compound **4d** but with a higher level of activity. Branching of the methyl moiety is unlikely and would cause a decreased level of activity. Similar structure–activity relationships among alkyl analogues (a decreased level of biological activity due to branching of the methyl moiety) were reported by Vinsova et al.[17] Unfortunately, compound R^1 = 4-Cl, $R^2 = CH_3$, which was expected to be highly active, was not prepared due to unexpected cyclization during the synthesis.[15]

All the compounds prepared in this study were screened at the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) run by The National Institute of Health of the US government.[18] The primary screening was conducted at 6.25 μg/mL (or molar equivalent of the highest molecular weight compound in a series of congeners) against *Mycobacterium tuberculosis* $H_{37}Rv$ (ATCC 27294) in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA). The compounds exhibiting fluorescence were tested in the BACTEC 460-radiometric system. The compounds showing <90% inhibition in the primary screen (MIC > 6.25 μg/mL) were not generally evaluated further.

Compounds **4i**, **4b** and **4h** showed interesting antituberculosis activity with inhibition levels in the range 89–99%, and the results are given in Table 1(B). The highest levels of activity against *M. tuberculosis* $H_{37}Rv$ were found for (*S*)-4-chloro-2-(4-trifluoromethylphenylcarbamoyl)-phenyl 2-benzyloxy-carbonylamino-propionate (**4i**, 99% inhibition, MIC = 3.13 μg/mL) and (*S*)-4-chloro-2-(3-chlorophenyl-carbamoyl)-phenyl 2-benzyloxycarbonylamino-propionate (**4b**, 97% inhibition, MIC = 3.13 μg/mL).

The levels of antituberculosis activity of compounds **4a**–**4j** given in Table 1(B) suggest that lipophilicity is again a secondary parameter for antituberculosis activity, and there is no correlation between log*k* and the antituberculosis activity of these compounds. Only the range of preferable hydrophobicity from 0.7 to 0.8 can be observed.

The type and the position of substitution are of great importance. The most appropriate is substitution on the $C_{(4)'}$ position by the CF_3 moiety.

The values of individual substituent electron-deficiency expressed as Hammett's parameter σ are connected with the above-mentioned facts. Hammett's constants σ_m for *meta* and σ_p for the *para* positions of the individual substituents were taken from the literature.[19]

The electron-withdrawing effect caused by 4-CF_3 substitution (σ_p = 0.54 for **4i**, **4h**) and/or 3-Cl substitution (σ_m = 0.37 for **4b**) seems to be the most important for antituberculosis activity. The optimum of electron deficiency, which corresponds to a higher level of antifungal activity, is approximately 0.5 and the range is 0.4–0.6. Any value outside that range causes a decrease in the level of activity; for example, 4-Cl σ_p = 0.23 for **4f** and 3,4-Cl σ_{p+m} = 0.60 for **4j**.

Low-bulky substituent, that is, methyl, or absence of R^2-substitution is another parameter for high antituberculosis activity. Substitution by a bulkier moiety than methyl, that is, isopropyl or benzyl, was associated with a significantly decreased level of activity.

A very important parameter influencing the activity is stereoisomerism, because individual enantiomers demonstrate considerable difference in their antituberculosis activity, for example, (*S*)-enantiomer **4e** showed much lower activity than (*R*)-enantiomer **4f** and on the contrary (*S*)-enantiomer **4b** showed much higher activity than (*R*)-enantiomer **4c**. The determined differences in antituberculosis activity for individual *R*/*S*-enantiomers cannot be explained on the basis of the results presented here. These facts are under intensive investigation.

On the basis of the facts discussed above, it can be assumed that the stereospecific bond can be probably formed between the compound and enzyme in *M. tuberculosis* with subsequent enzyme inhibition.

In summary, a new type of salicylanilide pro-drug was designed and several representatives were synthesized. The series was screened for antituberculosis and antifungal activity. Most of the tested compounds possessed a high level of in vitro antituberculosis activity. An antifungal assay showed levels of activity against *Aspergillus fumigatus*, *Absidia corymbifera* and *Trichophyton mentagrophytes* similar to that of fluconazole. Relationships between structure and biological activity, including the experimentally determined degree of liphophilicity, are discussed.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.080.

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