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Solid-phase synthesis and anti-infective activity of a combinatorial library based on the natural product anisomycin

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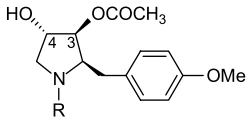
Abstract—The solid-phase synthesis of a library based on the natural product anisomycin is described. The resulting library was tested against a panel of bacterial and fungal targets, and active compounds were identified in a *Staphylococcus aureus* whole-cell assay and an efflux-deficient fungal whole-cell assay.

Antibiotic resistance is increasing at an alarming rate and the discovery of antibiotics to treat new resistant strains has not kept pace. There are both scientific and market reasons for this decline in new antibiotics. Most traditional antibiotic discovery efforts have targeted only a subset of the potential targets available and—due largely to limitations in screening throughput—have not explored the wider chemical and biological space. However, the advent of whole genome analysis can be expected to provide a plethora of new antibiotic targets. Another factor affecting the discovery of novel antibiotics has been the general decline in natural products research and screening[1] even though roughly 60% of the currently marketed antitumor and antibiotic agents are of natural products origin.[2] In part, this decline can be attributed to concerns over intellectual property (ownership of naturally occurring samples can be difficult to establish) and hit deconvolution (isolating and identifying the active component of a natural product mixture is a time-consuming and high-risk venture). The synthesis of high-purity combinatorial libraries based on a natural product template provides a unique opportunity to generate new 'natural product-like' samples while avoiding the aforementioned limitations. Herein we report the identification of novel antibiotic and antifungal compounds from libraries based on the natural product (−)-anisomycin (**1**).



1 R = H [(-)-anisomycin]

2 R = Fmoc [Fmoc-anisomycin]

A number of natural products based on the polyhydroxylated pyrrolidine nucleus have been identified as therapeutic agents for the treatment of protozoan and fungal infections, as well as diabetes and AIDS.[3] Of these, anisomycin (**1**) is an antibiotic that was first isolated from two *Streptomyces* species in 1950s.[4] It is clinically useful for the treatment of both amoebic dysentery[5] and trichomonas vaginitis,[6] and as a fungicide to eradicate bean mildew.[7] Recently, anisomycin has received attention as a potent in vitro antitumor agent with IC_{50} values in the nanomolar range.[8] Similarly, the combination of anisomycin and a cyclin-dependent protein kinase inhibitor has been shown to kill carcinoma

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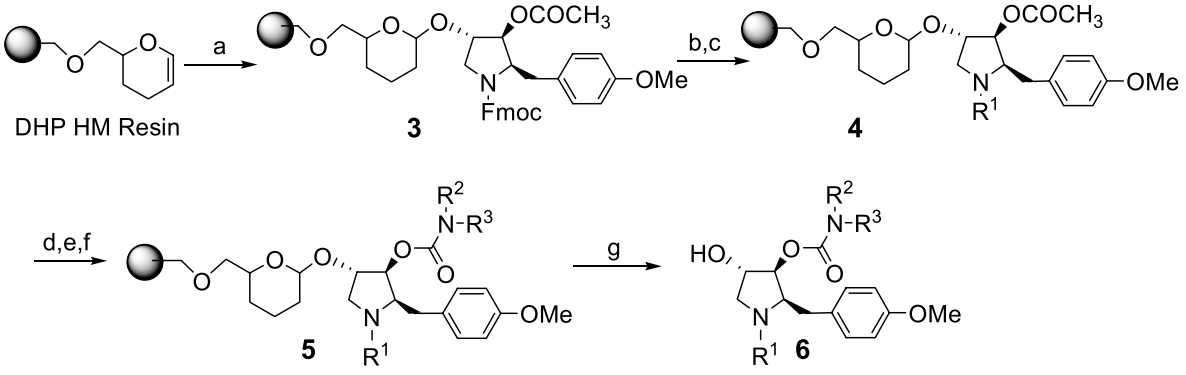
cells.[9] In eukaryotes, anisomycin exerts its cytotoxic effects through the inhibition of peptidyl transferase in the 60S ribosomal subunit[10] and the activation of JNK and p38 kinases.[11] In addition to these interesting biological activities, the methylene-linked five-six ring system of anisomycin is a common molecular framework for known drugs.[12] These pharmacological properties prompted us to investigate methods for generating a large and diverse set of anisomycin derivatives by solid-phase combinatorial chemistry. The resulting anisomycin library was expected to contain novel natural product-like samples with antibiotic activity.[13]

Our approach to generate an anisomycin library via solid-phase synthesis is shown in Scheme 1. We selected to link the C(4) alcohol of a suitably protected anisomycin derivative to the solid support using Merrifield resin equipped with a dihydropyran linker (DHP HM resin). Thus, anisomycin was treated with Fmoc-OSu and triethylamine in acetonitrile to give Fmoc-anisomycin (**2**) which was subsequently loaded onto DHP HM resin via Ellman's method[14] to provide **3**. Some modifications were necessary for this reaction. To conserve our limited supply of Fmoc-anisomycin, the ratio of **2** to resin loading was reduced to two equivalents and reaction time was extended to 48 h. The Fmoc group of resin **3** was removed by treatment with 20% piperidine in DMF and the first element of diversity (R^1) was introduced at the pyrrolidine nitrogen via reaction with acylating agents, sulfonyl chlorides, isocyanates, chloroformates, and isothiocyanates to provide the corresponding amide, sulfonamide, urea, carbamate, and thiourea derivatives (**4**). The acetyl group of resin **4** was then hydrolyzed using sodium methoxide in THF. The liberated C(3) hydroxyl group was treated with 4-nitrophenyl chloroformate and triethylamine in DCM to form the corresponding 4-nitrophenyl carbonate which, upon treatment with primary or secondary amines, afforded carbamate **5** with a second element of diversity (NR^2R^3). The resulting anisomycin derivatives were cleaved from the resin by the treatment of TFA/DCM/MeOH (2:2:1) at RT for 1 h, then the resin was filtered off and solution was collected in 96-well plates. After the solution was removed by speedvap, products **6** were obtained without further purification. Purity of products was identified by HPLC at 254 nm and mass spectroscopy, quantity of products was determined by Bohdan weight automation.

Prior to embarking on the synthesis of a large library, Scheme 1 was validated with a wide variety of building blocks. For the introduction of the first element of diversity (step c), acid chlorides, chloroformates, isocyanates, and carbamoyl chlorides were well tolerated under standard conditions. Aromatic sulfonyl chlorides worked very well, but aliphatic sulfonyl chlorides failed to afford the desired product under standard conditions—no reaction was observed. Isothiocyanates provided the desired product by mass spectroscopy, but only a low percentage of reagents provided acceptable purities (>70% by HPLC at 254 nm) due to poor conversion. For the introduction of the second element of diversity (NR^2R^3, step f) both primary and secondary amines worked very well, but anilines did not provide desired products under standard conditions—no reaction was observed. Based on the wide commercial availability of building blocks, we selected to synthesize a library comprising 10,000 members with dimensions of 100 $R^1 \times 100$ NR^2R^3.

In contrast to the ready availability of the aforementioned R^1 and NR^2R^3 reagents, commercial supplies of anisomycin were severely limited (and expensive, on the order of $1000/g). Fortunately, we were able to access large quantities of anisomycin via fermentation with an in-house isolated *Streptomyces* strain. Two tank fermentations of this strain, each with 3000 L of the production medium, were carried out in batch mode at 28 °C and 155 rpm for 4 days with an aeration rate of 1 vvm (volume of air added relative to liquid volume per minute). The pH of the fermentation broth was controlled to be not higher than 8.0 with 30% sulfuric acid. The resulting culture broths were processed by extraction with *n*-butyl acetate, aqueous back-extraction (acid–base transfer), solvent partition and crystallization. A total of 360 g anisomycin with purity >90% were isolated. This material was converted to Fmoc-anisomycin (**2**) as described previously. With an ample supply of **2** on hand, we were able to rapidly synthesize ten thousand anisomycin analogs as described in Scheme 1. Samples which did not meet our purity criteria (product peak $\geqslant$70% by HPLC at 254 nm) were discarded. Over 8000 anisomycin deriv-



Scheme 1. Reagents and conditions: (a) **2** (2 equiv, 0.2 M)/PPTS (2 equiv)/1,2-dichloroethane, 80 °C, 48 h; (b) 20% piperidine/DMF, rt, 1 h; (c) acyl chloride (10 equiv), or sulfonyl chloride (10 equiv), or carbamoyl chloride (10 equiv), or chloroformate (10 equiv), or isocyanate (10 equiv) or isothiocyanate (10 equiv)/Et_3N (20 equiv)/DCM, rt, 18 h; (d) NaOMe (20 equiv)/MeOH/THF, rt, 6.5 h; (e) 4-nitrophenyl chloroformate (10 equiv)/Et_3N (20 equiv)/DMC, rt, 20 h; (f) 1° or 2° amine (10 equiv)/DMC, rt, 18 h; (g) TFA/DMC/MeOH (2/2/1), rt, 1 h.

atives were ultimately submitted for screening, and thus the submission rate for the library exceeded 80%.

The completed library was tested against a panel of bacterial and fungal targets. The panel included whole-cell assays (both bacterial and fungal) as well as biochemical assays using purified components. The bacterial screens were directed against *S. aureus* and measured the growth inhibition of liquid cultures via optical densitometry. A number of anisomycin derivatives were active against *S. aureus* as shown in Table 1, and **6a**, **6b**,[15] and **6c**[16] are representative examples with minimum inhibitory concentrations (MICs) between 16 and 32 μg/mL. In contrast to anisomycin itself (**1**), these samples exhibited dramatically reduced cytotoxicity against HEK293 mammalian cells. A basic amine appears to be required for *S. aureus* activity: samples **6d** and **e** are close analogs of **6a** and **b**, respectively, which lack a basic amine and do not inhibit *S. aureus* growth. The fungal targets were limited to growth inhibition screens of *Candida albicans* (*C. albicans*, both efflux-deficient and ERG1 strains). Growth inhibition was measured by fluorescence of the growth indicator alamarBlue™ for the efflux-deficient strain, and optical densitometry for the ERG1 mutant strain. As shown in Table 2, structures **6f–k** were active in the efflux-deficient fungal whole-cell assay affording percent inhibitions between 80% and 95% at a concentration of 16 μM again with no significant cytotoxicity in HEK293 cells. The high degree of homology observed with **6f–h** and **6i–k** is quite impressive, considering that these samples were identified from a set containing over 8000 closely related analogs. The biochemical screens were directed against the enzymes of the cell wall biosynthesis pathway (MurPath and MraY/MurG). Details of the MraY/MurG screen can be found elsewhere[17]—no active samples were identified from the anisomycin library. The mechanisms of action for these bacterial and fungal growth inhibitors have not yet been determined, but all nine samples (**6a–c**, **6f–k**) were selective for their respective targets (and not cytotoxic against mammalian cells) which suggests that a cell-specific interaction is responsible for the activity.

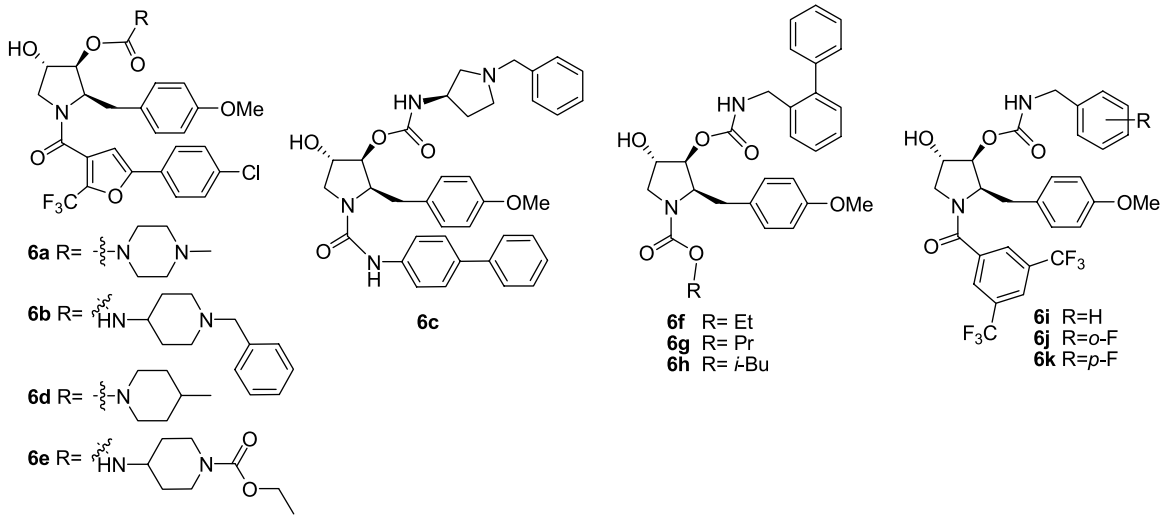


Table 1. Active samples identified in *S. aureus* assay

	S. aureus MIC (μg/mL)	MurPath Percentage inhibition at 25 μM	MraY/MurG Percentage inhibition at 25 μM	HEK293 cytotoxicity IC_{50} (μM)
1	>32	Not tested	Not tested	0.02
6a	32	<5	10	10
6b	16	<5	<5	8
6c	16	<5	20	2

Table 2. Active samples identified in efflux-deficient *C. albicans* assay

	Efflux-deficient *C. albicans* Percentage inhibition at 15.5 μM	RG-1 *C. albicans* Percentage inhibition at 15.5 μM	HEK293 cytotoxicity IC_{50} (μM)
1	>99	Not tested	0.02
6f	83	14	22
6g	96	10	9
6h	82	<5	11
6I	87	8	16
6j	87	<5	10
6k	87	<5	8

In summary, we have developed an efficient solid-phase method for the synthesis of anisomycin derivatives. Samples from the library were active in a *S. aureus* bacterial whole-cell assay and a *C. albicans* fungal whole-cell assay, supporting our hypothesis that a combinatorial library based on a known antibiotic template would afford novel antibiotics.

Acknowledgments

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15. ^{1}H NMR data for **6b** (spectrum collected at 120 °C due to amide rotomers): (500 MHz, DMSO-d_6, δ): 1.76 (m, 2H), 1.95 (m, 2H), 2.85 (m, 3H), 3.22 (m, 2H), 3.37 (m, 1H), 3.58 (m, 3H), 3.73 (s, 3H), 4.10 (m, 3H), 4.51 (m, 1H), 4.82 (m, 1H), 6.81 (d, J = 8.5, 2H), 7.02 (s, 1H), 7.11 (d, J = 8.5, 2H), 7.43–7.46 (m, 5H), 7.55 (d, J = 8.5, 2H), 7.79 (d, J = 8.5, 2H). Exact mass for $C_{37}H_{37}ClF_3N_3O_6$: 711.2323 (calculated), 711.2319 (found).
16. ^{1}H NMR data for **6c**: (500 MHz, MeOH-d_4, δ): 1.73–1.83 (m, 1H), 2.18–2.33 (m, 1H), 2.65–2.88 (m, 3H), 2.95–3.15 (m, 3H), 3.30–3.39 (m, 1H), 3.5–3.65 (m, 1H), 3.43 (s, 3H), 3.81–3.95 (q, 2H), 3.95–4.01 (m, 1H), 4.06–4.16 (m, 1H), 4.41–4.52 (m, 1H), 4.63–4.71 (m, 1H), 6.67–6.69 (d, J = 8.5, 2H), 7.02 (s, 1H), 7.09 (d, J = 8.5, 2H), 7.18–7.23 (m, 1H), 7.24–7.41 (m, 5H) 7.43–7.48 (m, 2H), 7.48–7.53 (m, 2H). Exact mass for $C_{37}H_{40}N_4O_5$: 620.2999 (calculated), 620.3008 (found).
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