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Use of combinatorial and multiple parallel synthesis methodologies for the development of anti-infective natural products*

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Abstract: In an astonishingly short time, combinatorial and multiple parallel synthetic methodologies for the synthesis of small drug-like molecules have transformed the practice of medicinal chemistry and are now in general use. Large focused and unfocused arrays of chemicals can now be produced and tested rapidly for the purpose of pharmacological evaluation. Rapid biological assays capable of performing tens of thousands of assays per week provide a driving force for the rapid generation of new chemical entities. Novel chemical strategies adapted to these purposes are represented by numerous research articles. The primary emphasis of much of this work has, however, been focused upon wholly synthetic substances. Whereas natural products can be considered to be nature's combinatorial libraries and continue to provide many important therapeutic substances, they are under represented for the most part in the literature of combinatorial chemistry. Indeed, there are those who believe that natural products are archaic in the face of these new methods. This paper addresses this question from the vantage point of representing the search for novel chemotherapeutic agents active against bacterial, fungal and viral pathogens by demonstrating that combinatorial and natural products methodologies are not antithetical but can be complimentary.

Arising from the needs of biochemists and molecular biologists less than 20 years ago, the ability to synthesize rapidly very large collections of related chemicals (called libraries) at first utilized Merrifield-like bead bound methodologies for the construction of peptides and then expanded to the production of arrays of oligonucleotides, lipids, and carbohydrates. From a comparatively slow beginning, this field has accelerated rapidly and numerous publications now appear weekly [1]. While useful for biological purposes, molecules such as these are generally useless for oral medication [2]. The first libraries of drug-like molecules appeared approximately five years ago in an attempt to deal with this problem [3].

When pharmacological testing was time consuming, preparation of molecules for biotesting could be carried out one at a time with careful attention to identity, purity, the likelihood of biological activity and the use of sophisticated and novel routes. In the classical mode, a skilled chemist generally produced about 50–100 molecules annually. This was sufficient to keep a group of biologists busy using classical pharmacological methodology. The development of automated high-throughput pharmacological screening methods now makes it possible to determine tens of thousands of data points weekly throwing the system out of balance since entire sample collections present in large firms having been accumulated over many decades could now be screened in short order. The demand for new substances to supply these new screens required the generation of newer methods of construction and provided the impetus for development of combinatorial methods whereby thousands of candidate substances could be prepared simultaneously if the appropriate chemistry were developed [4]. For these purposes, the numbers of compounds in libraries should be large, molecularly diverse and reasonably drug like. The chemistry is

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most often bead based, robotized and the individual compounds are analyzed through sampling selected individuals rather than comprehensively and small amounts of each substance are produced. Initially tested in mixtures, the number of false leads produced in this manner has now generally resulted in testing as individual samples. Novel chemical procedures have been developed for producing the library contents, rather expensive robotized equipment facilitates the construction and decreases the potential for human error, ingenious methods have been developed for coding the contents of individual beads, micro methods for the analysis of the identity and purity of individual beads are available and data bases have been developed for construction of virtual libraries and for keeping track of the multitudes of pharmacological data points. Companies have sprung up which construct libraries on assignment or on speculation and provide the equipment for this work. Increasingly, however, firms have brought combinatorial chemistry inside so the boutique firms specializing in these methods increasingly now are asked to provide specific leads rather than collections of chemicals or specialized chemical expertise.

Smaller, more focused libraries consisting of hundreds of samples in milligram quantities are utilized once a hit has progressed to lead status. This progression requires prior confirmation of activity in whole cells, organ systems or animals, reasonable potency, acceptable specificity, and assurance of reasonable freedom from toxicity. Libraries are constructed to determine the relationship between structure and pharmacological action through identification of the pharmacophore. Although bead based chemistry can still be employed, solution based chemistry is increasingly popular for these libraries. Next, smaller focused libraries are constructed in order to identify compounds with sufficiently attractive biological activity to justify the preparation of quite significant quantities for extensive biological evaluation and to provide repository material for archiving. Beyond this stage, the project usually returns to the use of traditional usual speed analoging (USA) methodologies with which the reader is likely to be quite familiar.

Underlying all of this work is the requirement that the contents of the libraries be drug-like and, for most modern therapeutic purposes, contain molecules capable of oral activity. Thus increasingly attention is being paid to an early incorporation of ADME (Absorption, Distribution, Metabolism and Excretion) considerations as experience indicates that a very large proportion of the drugs which fail in the clinic do so because of deficiencies in these characteristics. By compounds with suitable ADME is meant substances which have a realistic chance of being absorbed from the gastrointestinal tract following oral administration, be distributed to the site of the pathology which needs repair, do not undergo extensive or rapid metabolic alteration to inactive, rapidly excreted or toxic products, and are excreted at an acceptable rate. An understanding of these factors is still In the early stages so much empiricism is involved in drug seeking. The Lepinski rules of five have become popular to guide this work [5]. Essentially, these rules suggest that the molecules have a molecular weight under about 500, have a calculated log P between -1 and $+5$, not donate more than five hydrogen bonds nor accept more than 5×2 hydrogen bonds. If a molecule fits all of these requirements it may still fail to be orally active but at least it has a reasonable chance of succeeding.

The field of combinatorial chemistry has now grown into such dimensions that it defies concise comprehensive review. We have previously published accounts of multiple parallel synthetic investigations for fluoroquinolone antibacterials [6,7] and of alkaloids for antitubercular therapy of infections due to multiply resistant micro-organisms [8]. There are many accounts of analogous studies [1]. These papers can be consulted for additional information. For the present purposes we have chosen to illustrate our approach, which is rather typical of many contemporary studies, by describing the operation of a screen established for the purpose of finding novel structures potentially useful for the treatment of systemic human fungal infections.

First our collection of about 1500 extracts of higher plants was screened for *in vitro* activity against *Candida albicans* using agar-dilution streak methods resulting in approximately 120 actives. The screen evolved during this study to incorporate the lessons learned. The active compounds were further examined against a battery of fungi to see whether these had usefully broad antifungal activity and to select those with fungicidal activity as most patients with systemic fungal infections are immune suppressed so are able to contribute comparatively little to their recovery. Next, to eliminate in so far as possible saponins and other detergent-like molecules, assays of hemolytic potential were performed using blood agar and release of ^{13}C labelled aminoisobutyric acid from fungal cells was also performed.

Compounds failing these tests would likely be active by nonspecific membrane disruption and thus be unlikely candidates for human systemic use because of toxicity and lack of specificity. Following this phase of screening, about 50 promising extracts remained. Existing antifungal agents used in the clinic are active by disruption of ergosterol-containing fungal cell membranes (amphotericin B), or ergosterol biosynthesis (the various conazoles and triazoles) or by inhibition of RNA function (5-fluorocytosine). We were primarily interested in discovering drugs with modes of action different from these, so the next tests in the evaluation cascade involved inhibition of glucan synthase, chitin synthase, fungal topolsomerases II and IV, and ergosterol biosynthesis. Only two related extracts remained of interest at this stage. Next we evaluated the inhibition of respiration of *C. albicans* and drug-induced alterations in the morphology of this pathogen. Finally, oral utility was examined in experimental animals who had been infected following inmunosuppression with *C. albicans.*

The application of this screen resulted in the identification of a group of antifungal neolignans. The compounds were present in extracts of *Krameria ixena* (from Puerto Rico) and *Krameria triandra* (from Peru) and were isolated by bioactivity-directed screening methods. They turned out to be previously known and their activity against *C. albicans* had previously been noted, Thus, no further interest would have taken place unless novel synthetic analogs of improved properties could be prepared for which patent protection would be possible. Their structures were of particular interest as they differed from all previously known antifungal agents and their molecular mode of action had not been uncovered. This combination of factors provided an excellent opportunity for development of a combinatorial library to address these questions and to compare this with compounds prepared by traditional synthetic methodologies. The usual speed analoging process and the more complex screening tests and extensive biological evaluations were performed in conjunction with scientists at Abbott Laboratories and will be published elsewhere. The remainder of this report will detail the combinatorial work.

The structure-activity relationships were revealed to a limited extent by examining the potencies of the natural substances. Kramerixin proved to be nearly equivalent in potency *in vitro* to amphotericin B (Table 1). Furthermore, its 2,4-dihydroxy arrangement in the pendant phenolic ring was important as deletions and methylations in the natural series dramatically decreased potency (Fig. 1).

Table 1 Comparison of antifungal spectrum of kramerixin and amphotericin B.

Organism	Kramerixin	Amphotericin B
Candida albicans	3.12	1.56
Candida kefyr	6.25	3.12
Candida tropicalis	3.12	0.78
Cryptococcus albidus	6.25	1.56
Torulopsis glabrata	3.12	3.12
Aspergillus niger	12.5	1.56

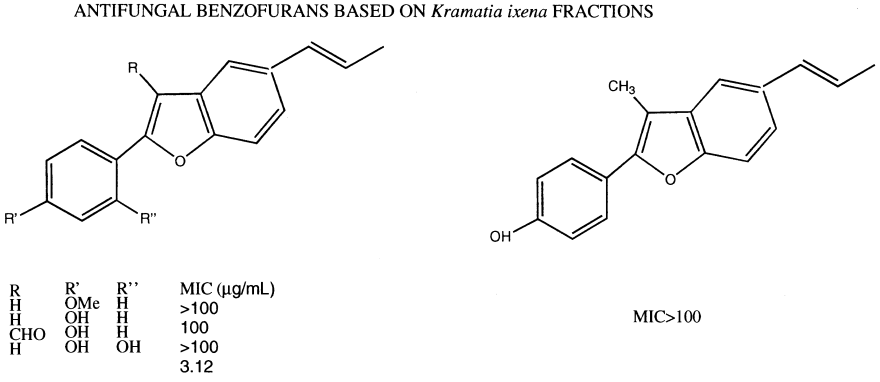


Fig. 1 Structures and antifungal activities of *Krameria neolignans*.

To work more systematically, however, required a convenient synthesis which could be employed in a combinatorial mode. Thus the molecule was figuratively dissected into two roughly equal halves involving readily available synthons. One part would stem from phenolic acids and the other from substituted phenols. Construction of the central furan ring or its equivalents would join these pieces through an ester Wittig reaction (Fig. 2).

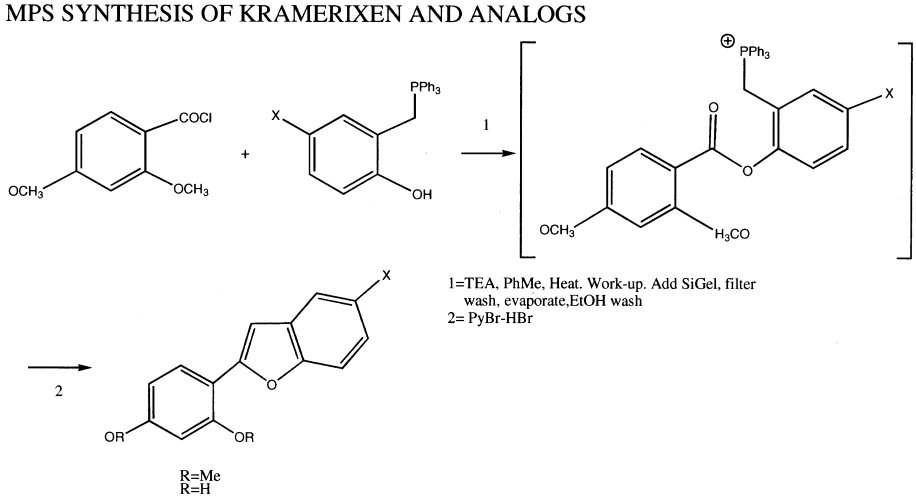


Fig. 2 Multiple parallel synthetic route to kramerixin and analogs.

Using a simple apparatus constructed inexpensively for these purposes [6], the requisite reaction conditions were ascertained rapidly through use of varying temperatures, solvents, times and modes of reaction (Table 2). Reduction to practice took place rapidly producing approximately 100 mg quantities of about 120 different chemically pure (microanalysis, NMR, MS and IR of each sample was ascertained) analogs were prepared and evaluated quantitatively for their antifungal potency.

Table 2 Development of workable reaction conditions for multiple parallel synthesis of kramerixin analogs.

Entry	Solvent	Temp (°C)	Time (h)	Yield (workup)
1	PhMe	Reflux	4	45% (usual)
2	PhMe	Reflux	4	31% (usual)
3	PhMe	Reflux	10	43%
4	PhMe	Reflux	14	37%
5	DMF	50–100	4	0%
6	Chfm	Reflux	4	0%
7	MeCN	Reflux	4	0%
8	THF	Reflux	4	18%

The structure–activity exploration utilized a figurative slicing of the molecule into three parts. In the first phase (examination of the proper number and arrangement of the substituent(s) W in box A) the benzofuran ring was kept constant and the pendant phenolic ring was varied. From this it was ascertained that nature had already selected the optimal arrangement of the phenolic hydroxyls.

In the second phase (examination of the nature of the heteroaromatic ring-X and Y in ring B above) it was learned that more structural variation was possible but that the furan ring was generally superior as compared with benzothiofurans, benzimidazoles, benzothiazoles and indoles. The indoles were better prepared using the classical Fischer indole synthesis than the Wittig chemistry illustrated above.

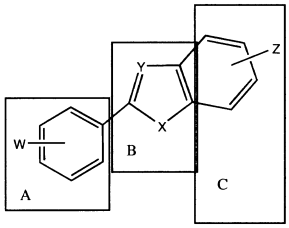
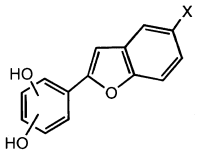
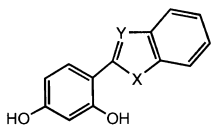
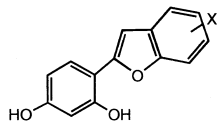


Fig. 3 Figurative dissection of kramerixin for structure–activity purposes.







A	B	C	
1,3-OH, X=M; mic 6.25	X=O, Y=CH; mic 6.25	X=CHCHMe, mic 1.56	X=CH_2NHMe, mic 100
1,3-OH, X=Cl; mic 3.12	X=S, Y=CH; mic 12.5	X=n-Pr, mic 3.12	X=NH_2, mic i
1,2-OH, X=Cl; mic 12.5	X=N, Y=CH; mic 25.0	X=Cl, mic 3.12	X=CH_2OH, mic 100
2,3-OH, X=Cl; mic 12.5		X=CH=NOMe, mic i	X=CN, mic i
2,4-OH, X=Cl; mic 25.0		X=CH_2Nme_2, mic 25	X=CHO, mic i
		X=CO_2Me, mic i	X=$CONH_2$, mic i
		X=H, mic 6.25	X=F, mic 12.6
		X=CH=CHPh, mic 12	X=5,7-diCl, mic 1.25
		X=NMe_2, mic 50	

Fig. 4 Structure–activity relationships of synthetic analogs of krarnerixin (the most active analog of 120).

In the third phase (examination of the optimal identity of Z in ring C) it was learned that Z is variable through a significant range of substituents. From this selection, the 5,7-dichloro analog (Fig. 5) and two related substances were chosen for *in vivo* evaluation following determination of their toxicity in mice.

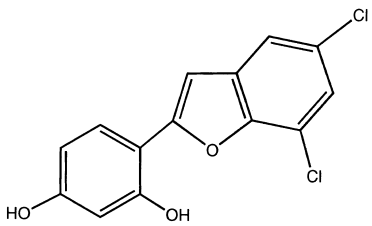


Fig. 5 The most active of the synthetic analogs of kramerixin.

Unfortunately, all three failed when tested orally at various doses in infected animals and the study was terminated at this stage for three reasons. First, the compounds, despite reassurance gained in phase 2 of the screening, produced detectable hemolysis raising questions about their safety. Subsequently, the criteria for passing this screen were tightened. Secondly, the compounds were found through electron microscopy to produce severe distortions of the morphology of *C. albicans* cells. Glucan 1,3-synthase is associated with the cell membrane and it is now felt that the inhibition of this enzyme by these agents is associated with allosteric effects secondary to more deep-seated alterations in the fungal cell so that this effect is not due to a specific inhibition of the enzyme itself. Finally, and most tellingly, the compounds were found to be strongly serum protein bound and failed to provide protection to artificially infected immune suppressed mice at nontoxic doses.

Although these agents ultimately failed, this saga illustrates the rapidity with which multiple parallel synthesis techniques allows one to reach a decision point in drug seeking. It also illustrates once again the

unfortunate fact that finding a drug suitable for the clinic is like winning a lottery. All of the numbers must be correct or one fails to earn the prize no matter how intensively or carefully one performs the necessary work. Clearly drug seeking is not for the easily discouraged.

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