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A search for novel chemotherapy against tuberculosis amongst natural products

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Abstract. Novel screens have been developed for the detection of antitubercular activity among natural product extracts and these were compared with existing methodologies. Using bioassay-directed methods these screens have been employed to isolate novel antitubercular natural products from higher plant extracts and their structures have been determined. In particularly promising cases, exemplified by tryptanthrin, an alkaloid from the Chinese herb *Strobilanthes cusia, i.a.*, conventional and combinatorial/matrix synthesis methodologies allowed the construction of hundreds of analogs in an attempt to optimize the activity. Tryptanthrin and its analogs are potent against multiresistant tuberculosis strains, are non toxic and give promising blood and tissue levels after oral administration to mice. The more potent of these, PA-505 and PA-510, are two orders of magnitude more potent than tryptanthrin itself and have been extensively evaluated *in vivo* but failed to cure infected mice. Nonetheless, the methodologies developed are promising for discovering novel natural products for evaluation against multiresistant tubercular and other bacterial infections.

At the beginning of this century tuberculosis was a devastating infectious disease, known as "the white plague"[1]. Mankind had no effective drugs to treat it so morbidity and mortality were high, especially among the poor. However, as the standard of living in wealthy nations trickled down to the urban poor and various preventive measures became widely practiced, the incidence of the disease fell. Better nutrition, less crowded living circumstances, pasteurization of milk, quarantine, use of sanitaria, prohibition of expectoration in public places, periods free from depression and war, and the like, all played a useful role. In the middle years of this century, a succession of effective antitubercular chemotherapeutic agents was discovered. The incidence of the disease continued to decrease until it largely faded from the public consciousness as a health hazard in industrialized nations. Consequently research on TB fell to a very low level in industry and academia alike. In recent years, this picture has changed significantly as many inner city neighborhoods deteriorate economically and populations almost everywhere continue to increase. The incidence of reported TB infections began to rise in the United States in the mid 1980s for the first time in this century. Homelessness, poor nutrition, crowded conditions, IV drug use, development of multiply drug resistant strains of bacteria, ease of international travel, and immune suppression due to AIDS and other causes are regarded as largely responsible [1]. Perhaps more disturbingly, the mortality rate (about 50% in the preantibiotic era; falling to about 15% in the antibiotic era with conventional TB) has increased to about 40% in immunocompetent patients with multidrug resistant TB (MDR-TB) and to 80% with MDR-TB in HIV patients. Thus MDR-TB in otherwise healthy persons has a mortality nearly equivalent to that in the preantibiotic era and in immunocompromised patients is actually significantly greater! Other infectious diseases are likewise becoming more resistant to chemotherapy so that a number of infectious disease experts fear a return to the perilous conditions of the preantibiotic era. Should anything like this occur, it will be recognized that we collectively have squandered a wonderful opportunity.

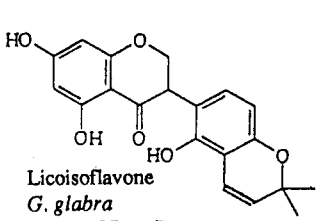
In poorer nations, the picture has been different. Here the story of this century has been less salubrious. Despite some spotty decrease in TB incidence, the disease has exacted a continuously terrible toll. Statistics demonstrate that despite a century of effort, today TB is the most lethal infection world wide due to a single agent, even surpassing malaria. There can be no doubt that a reinstitution of preventive measures and the discovery of effective new agents are urgently needed in order to deal with this situation.

Our laboratory is addressing this situation and finds that higher plant extracts are promising sources of novel antiTB leads.

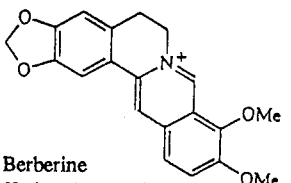
SCREENING Screening directly against tuberculosis is hazardous because of the highly infectious nature of the pathogen and is tedious because the organism is comparatively slow growing [2]. Our initial experiments employed *Mycobacterium smegmatis* as a surrogate test organism [3]. This organism is sensitive *in vitro* to the action of many established antitubercular antiinfectives, grows fairly rapidly and does not normally infect healthy humans. What was not clear at the outset is how accurately its use would reveal activity against MTB itself. To examine this question, we screened 1248 plant extracts at 1000 and 100 mg/L and found 236 (19%) to show significant inhibition. Through the courtesy of Dr. Scott Franzblau of Louisiana State University a selection of 33 of these were rescreened against *Mycobacterium tuberculosis* and *M. avium* complex using BACTEC technology [4]. This demonstrated that 20 (65%) were confirmed in this better established screen. Of these, 8 (25%) were active against both MTB and MAC, 8 were active primarily against MTB alone, 3 (10%) were active primarily against MAC alone and 13 were significantly less active. The BACTEC technology employs a special incubator containing a solution of radioactive fatty acid which is converted to radioactive carbon dioxide when the test microorganism metabolizes it. This radioactivity can be detected sensitively and much more quickly than can the growth of the organism by traditional visual means. The BACTEC is, however, expensive, the equipment is comparatively hard to obtain, the method does not allow for high throughput screening, and the radioactive waste presents a disposal problem.

As a screening alternative we have more recently developed and employed a novel screening procedure based on transfection of luciferase genes into strains of recombinant Bacillus Calmette et Guerin (BCG) and r*Mycobacterium intercellulare* [5]. These strains emit fluorescence in the presence of luciferin (The Lux Assay). When these strains are killed by an antibacterial agent, the lights literally go out. A reduction in relative light units (RLU) of 99% is considered active. Because of quenching exhibited by high concentrations of many of the extracts, maximum levels of 300 and 100 mg/L of extract were employed. Of 480 plant extracts examined, 16 (3.3%) were active. The utility and concordance of the assay was established by micro dilution assay comparisons and "seeding" with INH where indicated. It was also demonstrated that although too many false positives were detected, only a comparatively few false negatives had been missed with the *M. smegmatis* based screen.

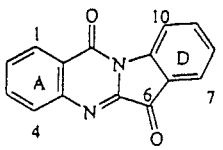
Antitubercular Natural Products Isolated Following These Technologies Among those extracts active in both the *M. smegmatis* screen and the confirmatory BACTEC screen against MTB and MAC, were the well known medicinal plants *Glycyrrhiza glabra* and *Hydrastis canadensis.* From *G. glabra* a series of flavanoids including the antitubercular agent licoisoflavone [figure 1] was isolated. It proved to be less active against *M. smegmatis* (50 mg/L) than MTB (25 mg/L). From *H. canadensis* the well known antibacterial alkaloid berberine was isolated [figure 1]. It was more active *in vitro* against *M. smegmatis* than against MTB (25 mg/L).



Licoisoflavone
G. glabra
MTB = 25 mg/L



Berberine
Hydrastis canadensis
MTB = 25 mg/L



Tryptanthrin
Strobilanthes cusia
MTB = 1 mg/L
MAC = 4 mg/L
MSmeg = 6 mg/L

Figure 1. Structures and potencies of some antitubercular natural products isolated using these methods.

A rather exciting result was obtained in exploring the constituents of the Chinese medicinal plant, *Strobilanthes cusia.* The active constituent turned out to be tryptanthrin. This substance was first prepared in 1915 by oxidation of indigo blue and isatin and by reaction of anthranilic acid with 2-chloroinden-3-one [6]. A number of more recent syntheses have been reported so the compound is now rather conveniently available [7,8,14,15]. It has also been isolated from indigoiferous plants, such as *Strobilanthes cusia, Polygonum tinctorium,* and *Isatis tinctoria*, which find folkloric use as topical fungicides [9-11], and in the

cannon ball tree, *Couroupita guianensis* [12]. Tryptanthrin and certain analogs are also prepared by directed biosynthesis using fermentation of the yeast, *Candida lipolytica,* to which a variety of synthetic tryptophane analogs were fed [13]. Thus tryptanthrin and some of its analogs were already well known as antiinfectives (although not as antituberculars) when we began our studies. Following up this lead, however, required dealing with freedom to operate considerations. In BACTEC studies (courtesy of Dr. Scott Franzblau), tryptanthrin proved rather more potent against MTB H37Rv (1 mg/L) and *M. avium* (4 mg/L) than against *M. smegmatis* (6 mg/L). This potency is in the same range as that of established antitubercular agents such as streptomycin, INH and ethambutol. Rather more excitingly, against multiply drug resistant (MDR) *M. tuberculosis* strain 10038, which was not affected by these three agents at comparatively high doses, tryptanthrin was equally active. One significant implication of this is that tryptanthrin can be proposed to be operating by a molecular mechanism different than that employed by most of the existing antitubercular agents and might be useful in cases where existing agents would fail to maintain or cure patients. Further encouragement was found when tryptanthrin was found to be little affected by serum protein binding and the bacteriostatic/bacteriocidal ratio was acceptable. The structure of tryptanthrin is comparatively simple, lacking asymmetric centers, it has a fairly low molecular weight and has a structure which differs significantly from all previously established antitubercular agents. Preparation of several analogs demonstrated that activity was preserved throughout a significant range of structural changes. Thus a more extensive analoging program was undertaken.

The existing chemistry of tryptanthrin was unsatisfactory for preparation of significant quantities and numbers of analogs needed for exploration of structure-activity relationships. This need was satisfied by extension of a synthesis developed earlier in this laboratory in which isatoic anhydrides are reacted with irreversible base and isatins [fig. 2][13,16]. This reaction figuratively dissects the molecule into two halves of similar synthetic complexity allowing preparation of a large and varied number of analogs for evaluation and, conveniently, can also be employed in combinatorial/matrix schemes of multiple simultaneous synthesis.

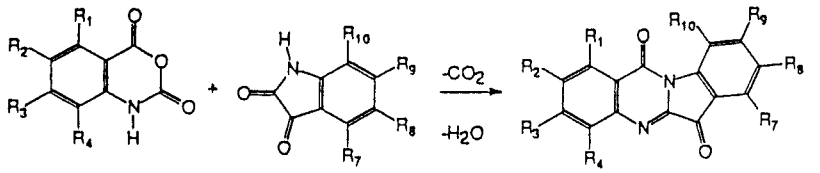


Figure 2. Synthesis of tryptanthrin used in these studies.

Using variants, several hundreds of analogs have been prepared and evaluated. These structures include many novel analogs not available by previous methods and a number of these are at least 100-fold more potent than tryptanthrin itself. The needed isatoic anhydrides (and anthranilic acid) halves can be made in a variety of ways of which lithiation and subsequent carbonation of suitable anilines is especially useful [3]. The needed isatin halves can be made by the classical Sandmeyer synthesis (reaction of anilines with chloral hydrate and hydroxylamine) or the more recent Gassman procedure (reaction of anilines with ethyl thiomethylacetate and t-butoxychloride followed by triethylamine and then oxidation with N-chlorosuccinimid and mercuric oxide) [17].

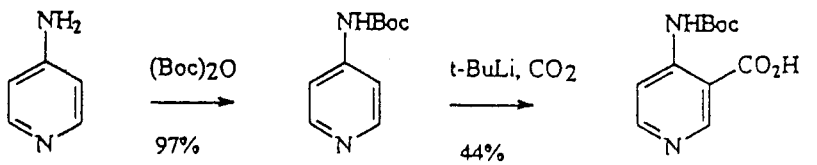


Figure 3. Carbonation of aminopyridine derivatives to make azaisatoic anhydrides.

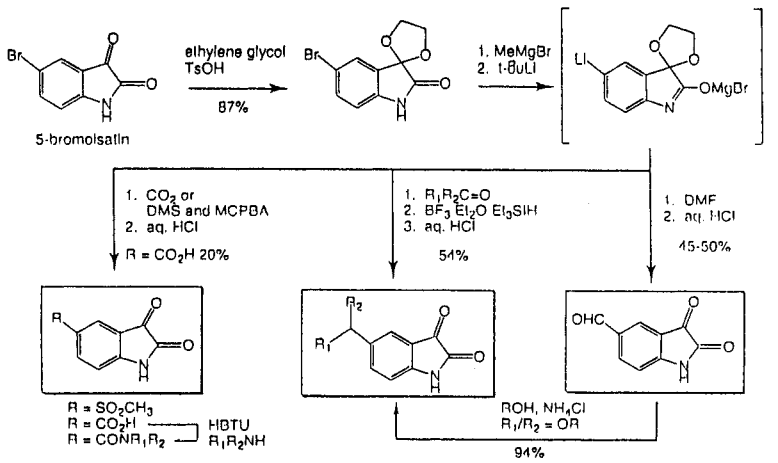


Figure 4. Further elaboration of isatins.

Additionally, when suitably protected as the dioxolane, halogenated isatins can be converted to their magnesium bromide salt with methyl Grignard and then metalated with t-butyl lithium. This product can then be carbonated, converted to a carboxaldehyde by reaction with DMF or alkylated with a ketone followed by reaction with boron trifluoride etherate and triethylsilyl hydride [fig. 4].

Further elaboration can take place at a later stage by displacing suitably arranged fluoro atoms from the tryptanthrin analogs using nucleophilic aromatic displacement protocols and suitable amines and thiols [fig. 5]. In some instances, the isatins can be transformed to the corresponding isatoic anhydride by careful oxidation procedures. Thus the chemistry described is particularly flexible and convenient for exploring the relationship between structure and activity in the tryptanthrin series.

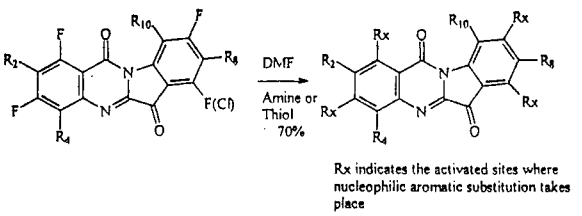


Figure 5. Further elaboration of tryptanthrin derivatives by nucleophilic aromatic displacement reactions.

Using this kind of chemistry, it was found that the C-6 carbonyl group of tryptanthrins was essential for bioactivity. Careful work with cyclic voltammetry demonstrated that this moiety was capable of entering into a redox cycle and that the oxidized form was the bioactive form. Furthermore, those analogs prepared which could not enter into redox cycles (the C-6-*gem*-dimethyl analog, for example) were inactive.

Many substitutions in the A ring were found to be compatible with powerful antitubercular activity [table 1]. After examination of a large number of analogs, the 2-aza family was found to be particularly promising [table 2].

Analogous substitution chemistry demonstrated that a wide range of substituents in the D ring was also compatible with powerful *in vitro* antitubercular activity [table 3].

A particularly effective combination of substituents is accomplished by aza substitution at positions 2 and 4 in the A ring along with various substitutions at the 8 position in the D ring [table 4 reflects the combination with C-2 substituted]. Some of these analogs are 100-fold more potent *in vitro* than

R_1	R_2	R_3	R_4	MIC (mg/L) against *M. Tb.* H37Rv
H	H	H	H	1.0
F	H	H	H	0.25
H	F	H	H	0.13
H	H	F	H	0.06
H	H	H	F	0.13
H	Cl	H	H	0.06
H	H	H	Me	0.13
Me	H	H	H	0.25
H	H	H	OMe	0.06
H	H	H	OH	0.25
H	NH_2	H	H	5.0

Table 1. *In vitro* antimycobacterial activity of various A-ring substituted tryptanthrins. The subscripts refer to the carbons involved.

A	B	C	D	MIC (mg/L) against *M. Tb.* H37Rv
H	H	H	H	1.0
N	H	H	H	8.0
H	N	H	H	0.25
H	H	N	H	1.0
H	H	H	N	0.25
N	H	H	N	5.0
H	N	H	N	4.0

Table 2. *In vitro* antimycobacterial activity of various A-ring azatryptanthrins

tryptanthrin itself and are patentably novel in their construction. Just as with tryptanthrin itself, these analogs generally retained their activity when tested against MDR MTB strains. An interesting trend is noted in that the activity increases with aliphatic substitution but plateaus after about C-4. Of the many analogs examined, PA-505 (2-aza-8-(2-octyl)tryptanthrin) was selected for detailed biological evaluation [fig. 6].

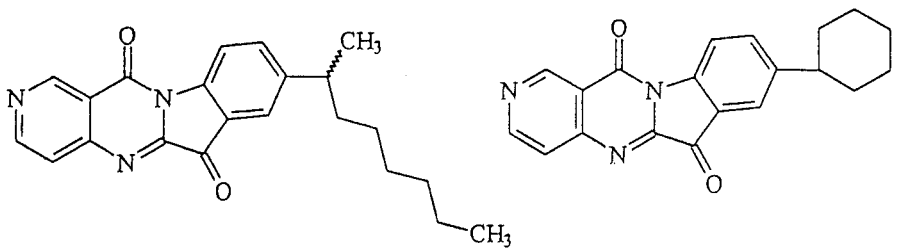
These analogs are generally approximately equally active against several sensitive strains of *M. tuberculosis* and several multiresistant strains. INH, on the other hand, loses at lease two logs in potency

R_7	R_8	R_9	R_{10}	MIC (mg/L) against *M. Tb.* H37Rv
Cl	H	H	H	0.5
H	Cl	H	H	0.06
H	H	Cl	H	0.13
H	H	H	F	0.25
H	F	H	H	0.13
H	I	H	H	0.13
H	NO_2	H	H	0.015
H	OMe	H	H	0.5
H	OCF_3	H	H	0.03
H	O-Octyl	H	H	4.0

Table 3. *In vitro* antimycobacterial activity of various D-ring substituted tryptanthrin derivatives.

C-8 substituent	MIC (mg/L) against *M. Tb.* H37Rv	MAC 19075
H	0.25	5
F	0.50	5
Cl	0.03	0.25
Br	0.03	0.25
I	0.03	0.25
CF_3	0.015	0.50
Me	0.13	1
Et	0.03	1
n-C_4H_9	0.015	0.06
n-C_6H_{13}	0.015	<0.2
n-C_8H_{17}	0.015	0.06
OCH_2OCH_2Me	0.06	1.0
n-C_8H_{17}	0.06	0.06
OCF_3	0.008	0.50
CHO	0.50	5
$CH(OCH_2)_2$	0.13	5
SO_2CH_3	4	>25

Table 4. *In vitro* antimycobacterial activity of some C-8 substituted 2-azatryptanthrin derivatives against *M. tuberculosis* H37Rv and *M. avium* (MAC) 19075.



PA-505 PA-510

Figure 6. Structures of synthetic antitubercular agents PA-505 and PA-510.

against these resistant strains. Since AIDS and immune suppressed patients bring little to no intrinsic resistance to the table it is considered important that novel agents be bacteriocidal in achievable doses. In the tryptanthrin series, analogs are occasionally cidal but are most commonly static. Nevertheless, the specific potency of many of the better analogs is sufficient for them to have a realistic chance to be bacteriocidal even if given alone. Against BCG (*M. bovis*) and Mtb H37Rv, INH has an MBC/MIC ratio of 1 (0.13/0.13) and 1 (0.05/0.03), which is particularly favorable. The corresponding ratios for tryptanthrin are 32 (8.0/0.25) and >128 (>128/1.0). PA-505 is 8 (0.13/0.016) against BCG and 133 (2.0/0.015) against Mtb H37Rv. Tryptanthrin is clearly a poorer choice for *in vivo* evaluation than PA-505. Despite the comparatively high ratio, the MBC dose is still only 2.0 for PA-505, which was judged to be an achievable blood level.

When given orally to mice, PA-505 gave much higher plasma drug levels than tryptanthrin (about 45% bioavailability) producing blood levels significantly above the mic/mbc level (PA-505 showed a peak plasma level of 3.5 mg/kg on day 8). At 5, 10 and 50mg/kg orally for 10 days, no apparent dose-related toxicity was observed. PA-505 penetrated quickly into the plasma (2.7-4.4mg/L in an hour), liver (7.1 mg/L), kidney (3.2 mg/L) and lung (2.0 mg/L). Somewhat lower heart (0.5 mg/L), spleen (0.6 mg/L) and brain (0.1 mg/L) concentrations were observed. Since MTB survives happily in macrophages, it was comforting to find that PA-505 was active in this environment although requiring somewhat higher doses (0.25 mg/L) than for INH (0.13 mg/L) at 3-8 days.

Analogs PA-505 and PA-510 were administered at comparatively high doses (50 mg/Kg) orally in infected mice. After 10 days of therapy, in comparison with INH, these analogs showed some slight efficacy

compared to controls, but failed to cure the animals. A similar failure with PA-505 given orally daily for 5 weeks or interperitoneally by infusion for 1 week was observed. The reasons for this disappointing outcome are not readily apparent but may be a consequence of the pharmacokinetic features being insufficient to overcome the comparative lack of bactericidal activity. This series has been terminated in favor of more promising substances. Efficacy in other disease situations has been observed for some members of this series and the results will be reported elsewhere in due course.

Despite the ultimate disappointment with the *in vivo* antitubercular properties of tryptanthrin analogs, it is clear that the methodology developed in this study works and that a wide variety of natural products structures possess potential utility for the treatment of multiply resistant bacterial infections if suitable methodology is employed. It is quite likely that promising substances lie presently untested in sample collections of phytochemists and their examination could repay the effort with striking findings.

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