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# Development of inhibitors of mycobacterial ribonucleotide Reductase

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New drugs for the therapy of leprosy have to be developed because of the small number of existing active drugs and because of the increasing resistance of *Mycobacterium leprae* against them.

One possibility to initiate the development of a new drug is to start with a known chemotherapeutic. Our project started with the investigation of a series of analogues of thiacetazone (*p*-AcNH-Ph-CH = NNHCSNH$_2$). Unfortunately no remarkable activity against our leprosy model strain *M. lufu* and several other mycobacterial strains could be found in the class of substituted benzaldehyde thiosemicarbazones (TSCs).

Quite unremarkable activities were also found for the first two heterocyclic TSCs in Table 1. But there is a considerable increase in activity in case of α-heterocyclic TSCs as shown by the third derivative.

Similar increases in activity on going from α-nonheterocyclic TSCs to α-heterocyclic analogues have been published for the antitumor potency of these compounds.[1] Several groups have shown that the antitumor activity of these α-heterocyclic drugs is caused by the inhibition of DNA synthesis.[2–7] Their site of action is the iron-containing enzyme ribonucleoside diphosphate reductase (RDR).

RDR together with the dithiolprotein thioredoxin promotes the reduction of ribonucleotides to deoxyribonucleotides which are essential intermediates in the synthesis of DNA (Figure 1).

By Sartorelli[2,8,9] some evidence has been provided that the non heme-iron atom in the active site of the reductase interacts with α-heterocyclic TSCs which are known to be tridentate chelators.

As it has been found that bacterial RDRs show some similarities to the mammalian enzyme[1,2,10–12] we suppose that the TSCs have the same mode of action in the antitumor test and in our antibacterial test system.


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**Table 1.** Minimum inhibitory concentrations (MIC) of pyridine-aldehyde-thiosemicarbazones towards *M. lufu*.

| R-CH = N-NH-CSNH$_2$ | MIC [$\mu$M/l] |
|---|---|
| R = 4-pyridyl | 611 |
| 3-pyridyl | 556 |
| 2-pyridyl | 40 |

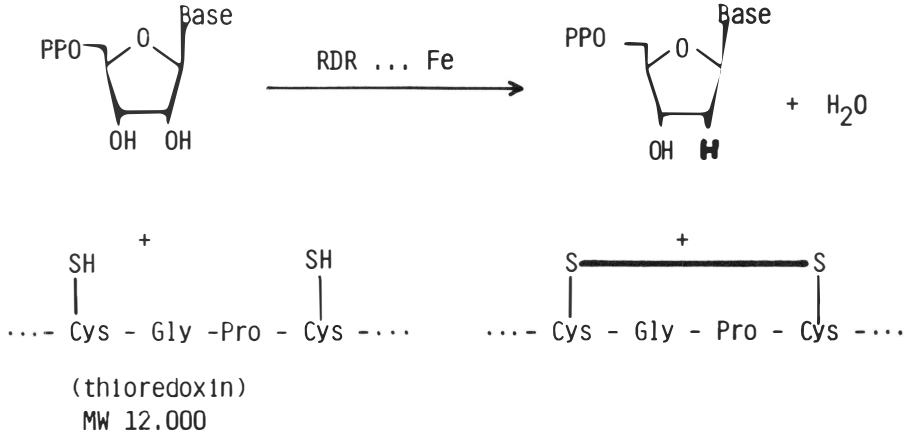


**Figure 1.** Reduction of ribonucleoside diphosphates by thioredoxin catalysed by the iron-containing enzyme ribonucleoside diphosphate reductase (RDR).

To investigate this hypothesis we tested a series of pyridine-2-aldehyde-TSCs and found that derivatives with substituents in the 6-position of the pyridine ring have low activities. As steric effects should be unfavourable for the formation of iron-complexes this result seems to support our hypothesis.

Several of our derivatives have also been tested by French and coworkers[1] for their inhibitory activity against RDR from human sarcoma cells.

A plot of the antimycobacterial potency of these compounds against their antitumor activity shows some colinearity (Figure 2) and therefore again seems to support the hypothesis of ribonucleotide reductase inhibition in *M. lufu*.

Pyridine-2-aldehyde-TSC derivatives are clearly more active against mycobacteria than thiacetazone, but unfortunately they are also clearly more toxic towards mammals (Table 3). The reason probably is increased delivery of toxic hydrogen sulphide.

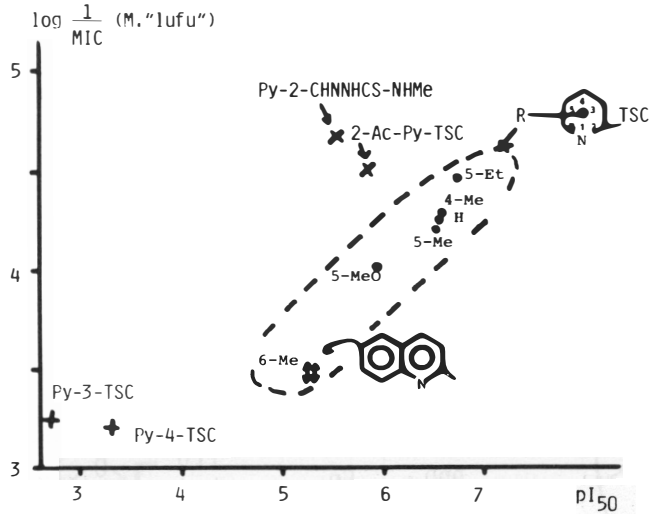


**Figure 2.** Plot of the antimycobacterial potency (log *1*/MIC, *M. lufu*) of acylpyridine-thiosemicarbazones *vs.* their antitumor activity ($pI_{50}$, in vitro inhibition of RDR from human epidermoid carcinoma).[1]

For this reason we started the investigation of a series of heterocyclic hydrazones which are not TSCs and cannot split off hydrogen sulphide. The general structure of these compounds is compared in Figure 3 with the previously discussed TSCs. For patent reasons the exact structure cannot be given.

Some antimycobacterial test results of two compounds of this series are shown in Table 2. From the MIC data it can be derived that these compounds are 20–30 times more active than thiacetazone (*M. lufu:* MIC = 340 $\mu$M/l) and 2 to 3 times more active than pyridine-2-aldehyde-TSC. Several compounds recently

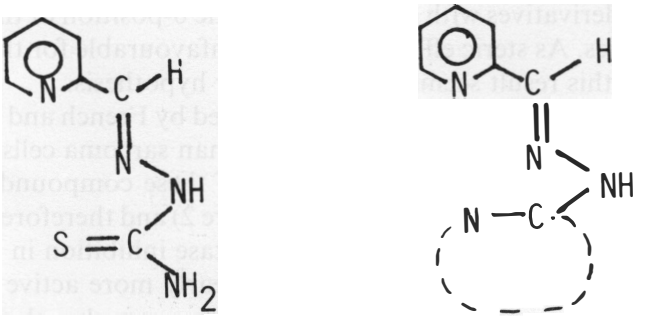


**Figure 3.** Structural comparison of two tridentate chelators: pyridine-2-aldehyde-TSC (left) and corresponding N-heterocyclic hydrazones of the type of the lead molecule PH22 (right).

**Table 2.** MIC values ($\mu$M/l) of two heterocyclic hydrazones towards mycobacteria

| | PH22 | PQ22 |
|---|---|---|
| *M. lufu* L209 | 18 | 10 |
| *M. smegm.* ATCC607 | 35 | 24 |
| *M. tbc* H37Rv | 80 | 43 |
| *M. avium* SN304 | 18 | 24 |
| *M. marinum* SN1254 | 18 | 12 |

developed in our laboratory from the new lead molecule PH22 show a further increase in activity by a factor of ten and have MIC values lower than 0·5 $\mu$g/ml. Both PH22 and PQ22 are known to be strong tridentate chelators of iron ions which are octahedrally surrounded by two ligand molecules.

As these heterocyclic hydrazones do not provide the possibility to split off hydrogen sulphide they can be expected to be less toxic than pyridine-aldehyde-TSCs. The results of the investigation of the acute toxicity of three compounds towards rats and mice are shown on Table 3. We found that the new lead compound PH22 is considerably less toxic than pyridine-2-aldehyde-TSC and is comparable with thiacetazone in this respect.

A very interesting aspect of hydrazones and TSCs is their synergistic antibacterial effect towards *M. lufu* in a combination with dapsone. By analysing

**Table 3.** Acute toxicity of PH22 (I), pyridine-2-aldehyde-TSC (II) and thiacetazone (III) towards rats and mice

| | $LD_{50}$ [mg/kg] | |
|---|---|---|
| (I) | $\geqslant 2000$ | rat, subcut.[1] |
| (II) | 30 | rat, subcut.[1] |
| (II) | 40 | mouse, i.p.[2] |
| (III) | 1000–2000 | mouse, subcut.[3] |

[1] Kazda, Schaper, unpubl. results

[2] French, Blanz. *Cancer Res*, 1965; **25:** 1454

[3] Bavin, Rees *et al. J Pharm Pharmacol*, 1950; **2:** 764

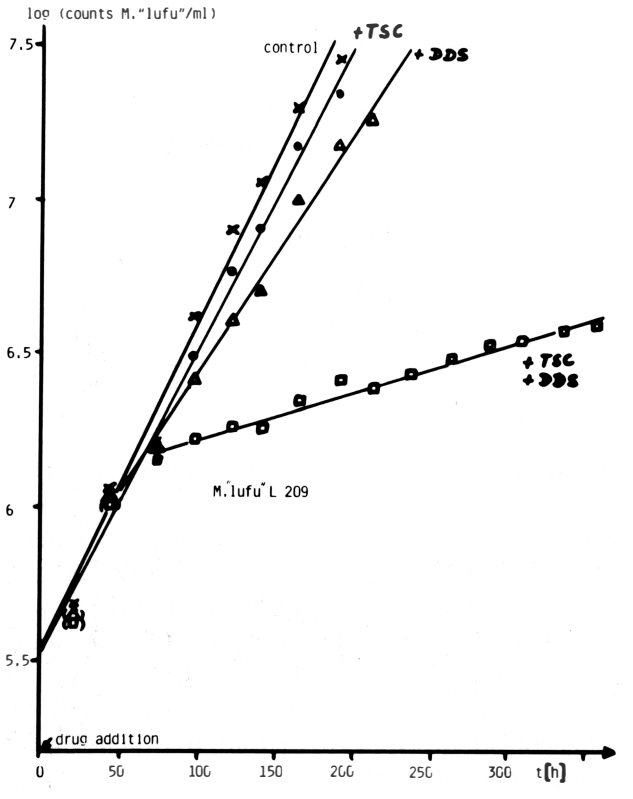


**Figure 4.** Bacterial growth kinetics of *M. lufu* at 31°C in the presence of pyridine-2-aldehyde-TSC and dapsone (DDS) alone and in combination at the concentrations indicated. x, control; ●, 5 $\mu$M/l Py-2-TSC; △, 0·2 $\mu$M/l DDS; □, 5 $\mu$M/l Py-2-TSC + 0·2 $\mu$M/l DDS.

bacterial growth kinetics[13] we found this potentiation of activity for both TSCs and heterocyclic hydrazones. Figure 4 shows that the control growth rate of *M. lufu* is reduced a little bit by low concentrations of pyridine-2-aldehyde-TSC or DDS. If both drugs are combined a clearly more than additive effect is obtained.

A very similar result is obtained if 2 to 3 times lower concentrations of PH22 are combined with DDS (Figure 5).

A possible explanation for the synergism is the fact that the synthesis of DNA is inhibited at two different sites of the DNA pathway: DDS is an inhibitor of folate synthesis[14] whereas the hydrazones seem to block deoxynucleotide synthesis (Figure 6).

One of the subsequent steps in the reaction sequence inhibited by dapsone can be blocked by trimethoprim (TMP) derivatives which are well known DHFR inhibitors.[15] If synergism is obtained by combining hydrazones and DDS then the

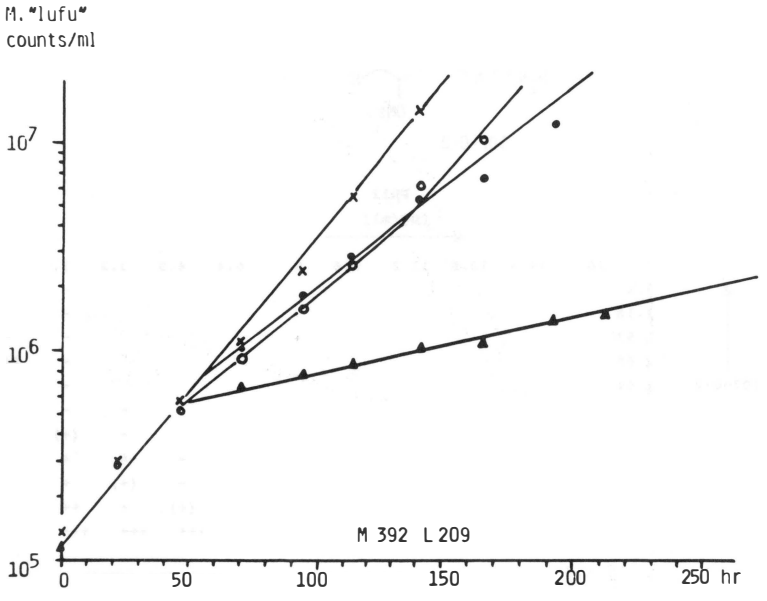


**Figure 5.** Bacterial growth kinetics of *M. lufu* at 31°C in the presence of PH22 and dapsone (DDS) alone and in combination at the concentrations indicated. x, control; ●, DDS 0·2 μM; ○, PH22 2 μM; ▲, DDS + PH22 0·2 μM + 2 μM.

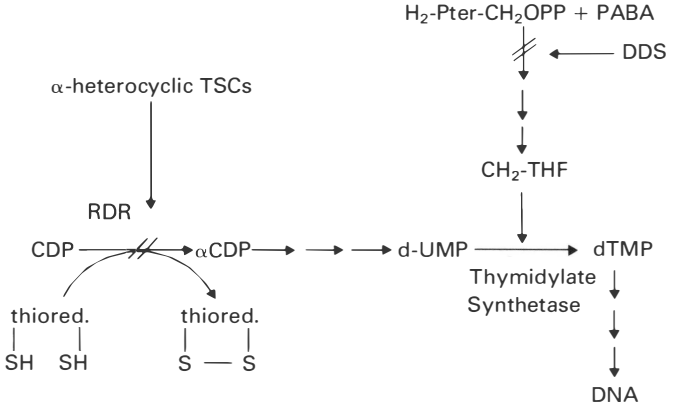


**Figure 6.** Sites of inhibition of DNA synthesis by sulphones (DDS) and hydrazones e.g. TSCs.

same effect should be observed in a combination of hydrazones and TMP derivatives.

This assumption is confirmed by Figure 7 showing for *M. lufu* the MIC determination by checkerboard titration[16] of combinations of PH22 with the TMP derivative 107-0-2. A clear synergistic effect can be recognized. An increase

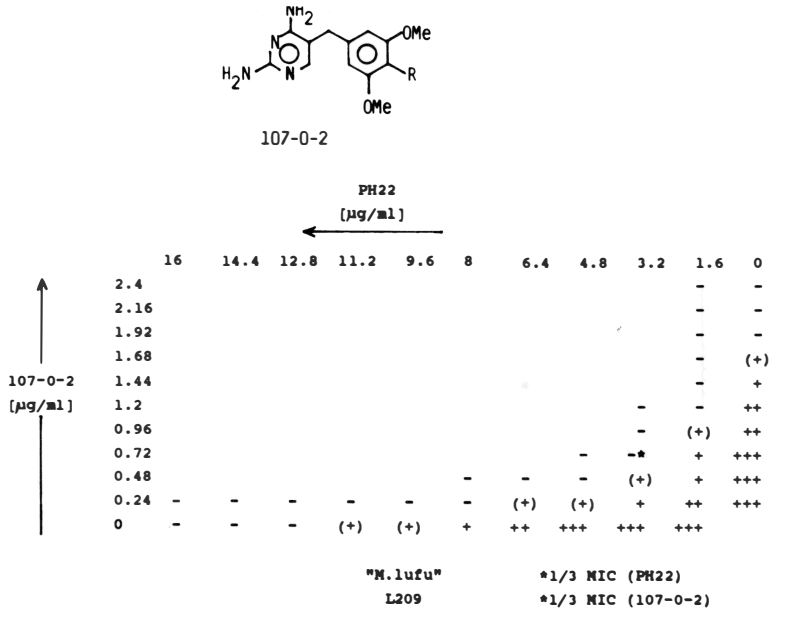


**Figure 7.** Antibacterial effects of PH22 and TMP derivative 107-0-2 alone and in combination on *M. lufu*. +, multiplication is observed; −, no multiplication observed.

in synergistic potentiation is obtained by combining PH22 with a TMP derivative which is more active towards *M. lufu*.

Fortunately a clear-cut further increase in the synergistic effect is observed (Figure 8) in a triple combination consisting of DDS and of PH22 + TMP 107-0-2 (which are applied in a fixed ratio of 5:1). Here a full stop of bacterial growth is obtained at concentrations of only 2% of the concentrations needed for single drug treatment in case of PH22 and TMP 107-0-2 and 25% in case of DDS.

And last but not least a very strong synergism is found for the combination of PH22 with the DNA synthesis inhibitor 5-fluoro-uracil. This antitumor drug is a blocker of the reaction catalysed by thymidylate synthetase (Figure 6) which promotes the methyl group transfer from methylene-THF to the 5-position of deoxyuridine monophosphate.[17]

So possibly PH22 derivatives are appropriate candidates for a multidrug therapy in leprosy.

The synthesis of DNA in mammalian and microbial cells depends on the rate limiting synthesis of deoxynucleotides which is catalysed by RDR.

As shown by Figure 9 the enzyme consists of two nonidentical subunits B1 and B2.[10] Each subunit by itself is completely inactive. At the active site subunit B2 contains two atoms of iron which are necessary for enzyme activity. Of similar importance is the presence of a tyrosine radical in the same subunit.

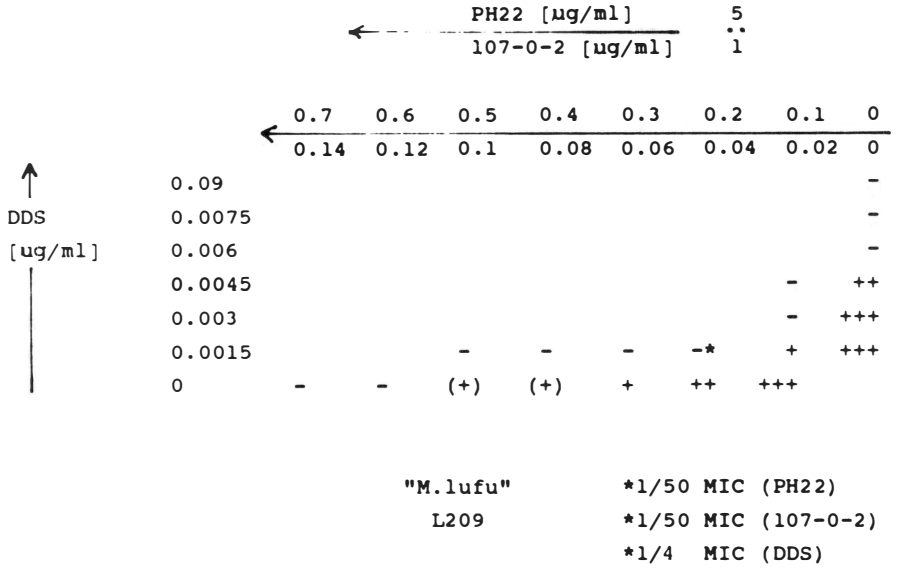


**Figure 8.** Antibacterial effects of PH22+TMP 107-0-2 (fixed ratio 5:1) and DDS on *M. lufu*.

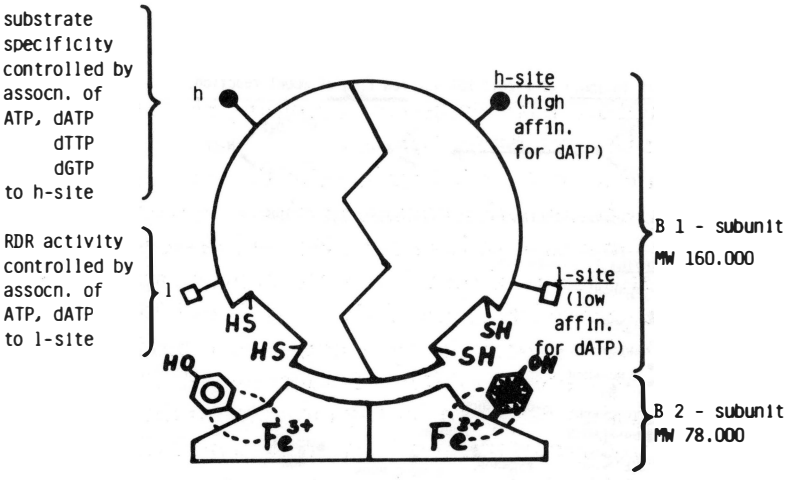


**Figure 9.** Thelander–Reichard model of ribonucleoside diphosphate reductase (RDR) from *E. coli*.[10]

In a simple chemical model system we tested the chelating properties of some of our derivatives and got for all of them almost identical results. So we had the impression that chelation of the iron atom of ribonucleotide reductase is not the activity determining step.

Several reports can be found in the literature[3,9,18,19] which seem to support this

conclusion and which suggest the active form of the inhibitor to be a preformed iron chelate. Interestingly also some ferrous ion complexes of our chelators were found to be quite active inhibitors of *M. lufu*.

As mentioned before the ribonucleotide reductase in addition to iron atoms also contains one tyrosine radical at the active site. The presence of the radical is closely linked to the presence of iron atoms. The radical is lost on removal of iron and reformed on reconstitution.[20] On the other hand the radical without loss of iron can be destroyed by hydroxylamine derivatives.[11,21]

Thelander found that there is some correlation (Figure 10) between the degree of inhibition of *E. coli* ribonucleotide reductase by N-hydroxyurea analogues and the corresponding rate of reaction of these compounds with an inorganic radical salt (Figure 10), which was considered to be a model system for the tyrosine radical. Thelander concluded that a very important parameter for the inhibitory potency of a drug is the ability to undergo a one-electron oxidation.[11,19]

It is well known that hydrazines and hydrazones are easily oxidizable compounds and that their oxidation often proceeds via radical mechanisms.[22–26] So one can conclude that the hydrazone chelators combine two molecular properties which may be important for activity: 1, the ability to form very stable chelate complexes; this seems to be a necessary prerequisite for activity; and 2, the

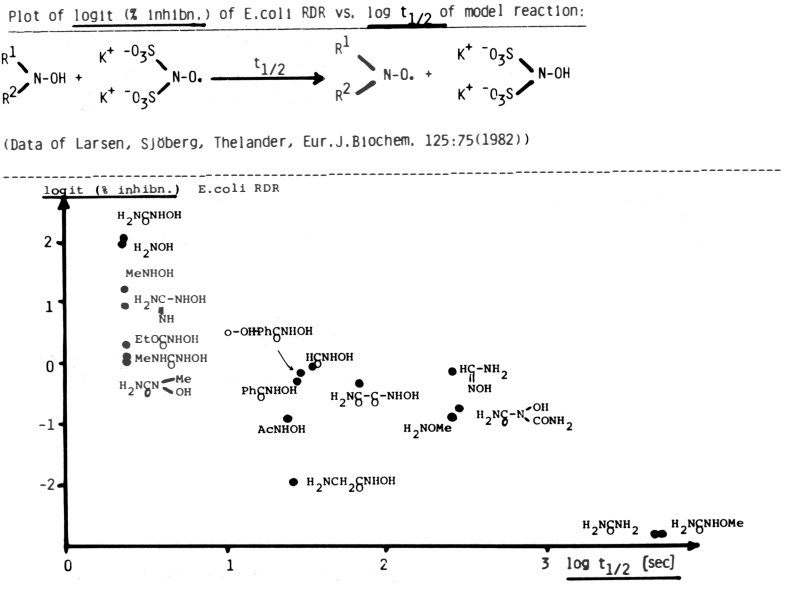


**Figure 10.** Comparison of inhibitory activity and reactivity of N-OH-urea derivatives; plot of log (% inhibition) of *E. coli* RDR *vs.* half life (log $t\frac{1}{2}$) of the model reaction shown at the top of the figure; data obtained from ref. (11).

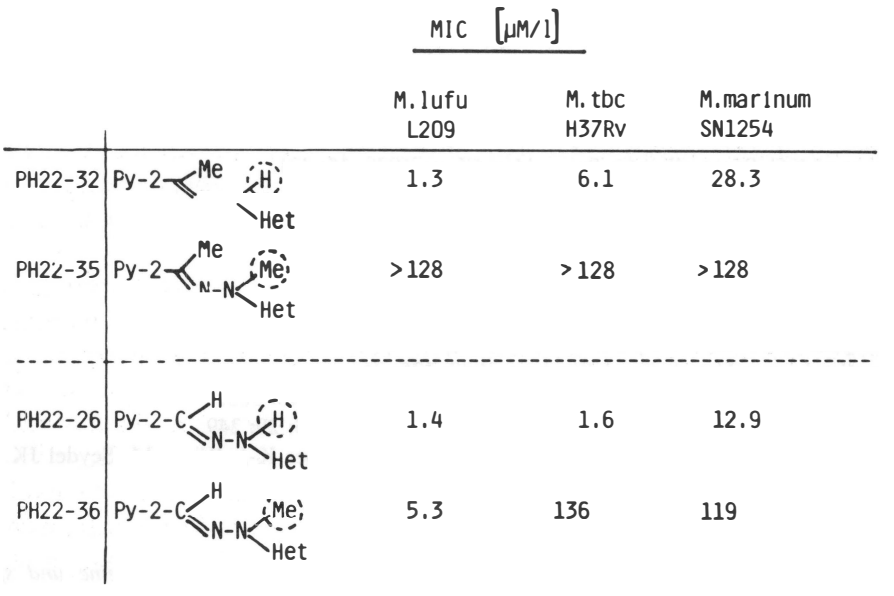


**Figure 11.** Decrease of antibacterial potency of PH22 derivatives observed after methylation of the hydrazone NH group.

ability to perform redox reactions; possibly the biological potency is modulated by the substituent effects on the postulated radical mechanism.

This last assumption is supported by the results shown by Figure 11. A large decrease in activity is observed if the most probable site of oxidative attack of the heterocyclic hydrazones is blocked by transforming the NH fragment within the hydrazone group into a N–Me group.

On the basis of the lead molecule PH22 we are presently trying to develop a new drug with high activity against leprosy by synthesizing a series of PH22 derivatives and testing their activity against mycobacteria. Unfortunately for patent reasons no details of this work can be given.

By applying quantitative structure–activity relationship analysis[27] we hope to extract relevant information for the attempted activity optimization.

## Acknowledgments

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