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New rifabutin analogs: Synthesis and biological activity against *Mycobacterium tuberculosis*

José Barluenga,[a,*] Fernando Aznar,[a] Ana-Belén García,[a] María-Paz Cabal,[a] Juan J. Palacios[b] and María-Angela Menéndez[b]

[a]*Instituto Universitario de Química Organometálica ''Enrique Moles'', Unidad Asociada al CSIC, Universidad de Oviedo, C/ Julián Clavería, 8, 33006 Oviedo, Spain*
[b]*Unidad de Referencia Regional de Micobacterias, Hospital Universitario Central de Asturias, C/ Doctor Bellmunt, s/n 33006 Oviedo, Spain*

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Abstract—The synthesis, structure, and biological evaluation of a series of novel rifamycin derivatives, Rifastures **(RFA)** with potent anti-tuberculosis activity are presented. Some of these derivatives showed higher in vitro activity than rifabutin and rifampicin against not only *Mycobacterium tuberculosis* strains but also against MAC and *Mycobacterium kansasii*.

The rifamycins are a family of naphthalenic ansamycin antibiotics of remarkable interest because of their structure, biogenesis, mechanism of action, and therapeutic efficacy.[1] They were isolated for the first time in 1959 from *Amycolatopsis mediterranei* as a mixture of at least five active substances, designated rifamycin A–E. Only rifamycin B was isolated as a pure crystalline substance, and it has the unusual property that in oxygenated aqueous solutions, it tends to change spontaneously into rifamycin S (Fig. 1), a compound with higher antibacterial activity.

Ansamycins are macrolide antibiotics characterized as having a 17-membered aliphatic bridge connecting two nonadjacent positions on a chromophoric naphthahydroquinone nucleus via an amide linkage,[2] and are generally active against Gram-positive bacteria and mycobacteria, especially *Mycobacterium tuberculosis*. Some rifamycins have interesting levels of activity against viral RNA-dependent DNA polymerase[3] but they are mostly known as potent inhibitors of all bacterial DNA-dependent RNA polymerases (DDRP).[4] It is believed that the mechanism of action of rifamycins involves primarily formation of a stable complex with bacterial RNA polymerase, binding with the β subunit of the enzyme, and effectively inhibiting RNA synthesis.[5] Most rifamycins are not effective on the mammalian RNA polymerase; therefore, they possess the necessary requisite of low toxicity.

Studies of structure–antibacterial activity relationships have shown the minimal requirements for activity to be the presence of oxygenated functions at the C-1, C-8, C-21, and C-23 positions, that are thought to be directly involved in the attachment to the enzyme.[6] Therefore, modifications at C-3 and/or C-4 positions, which are on the opposite side of the aromatic ring, have been mostly exploited for the preparation of new active derivatives. It is believed that these structural changes do not affect the antibiotic mode of action, but they are able to enhance activity by improving membrane permeability and pharmacokinetic properties.[7] Among all new products, Rifampicin (Fig. 1) (U.S. generic name is rifampin)[8] has been introduced into therapy for the last 30 years as a first-line agent against tuberculosis.[9]

Spiropiperidyl-substituted rifamycin derivatives[10] are a class of semisynthetic rifamycin antibiotics[11] in which the C-3 and C-4 positions have been incorporated into an imidazolyl ring bearing a spiropiperidyl group. The nitrogen of the piperidyl ring can be substituted with linear and branched aliphatic chains.[12] Among them,

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* Corresponding author. Tel./fax: +34985103450. e-mail: barluenga@uniovi.es

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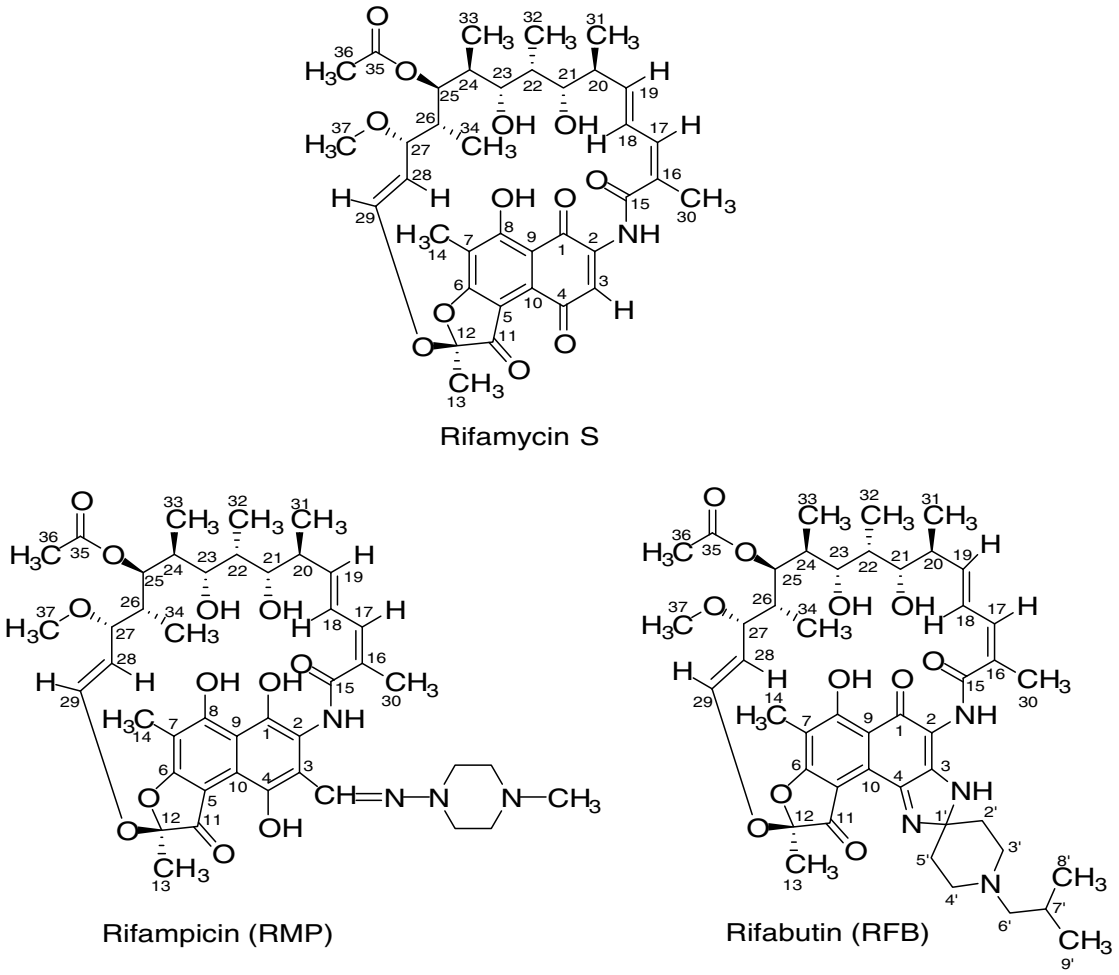


Figure 1. Chemical structure of some rifamycins.

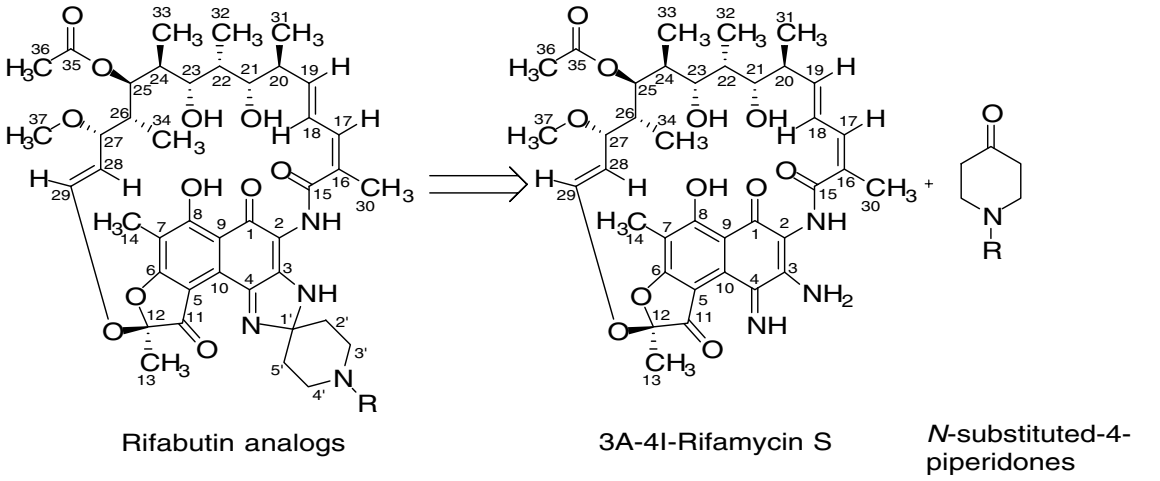
rifabutin[13] (Fig. 1) is an alternative agent[14] for the treatment of several mycobacterial infections, including both prophylaxis or therapy against disseminated MAC (*Mycobacterium avium-intracellulare* complex) infections in AIDS patients, and multidrug-resistant tuberculosis (MDR-TB) strains.[15] Unfortunately, rifabutin presents a high level of toxicity in vivo.

Recently, organizations such as the World Health Organization (WHO)[16] and the International Union against Tuberculosis and Lung Disease[17] have promulgated efforts to develop new drugs that will shorten the treatment duration and improve the therapeutic response of MDR-TB. In this paper, we report the synthesis, structure, and bioactivity of seven new rifabutin analogs, called Rifastures (**RFA-3**),[18] which show a high level of activity against *M. tuberculosis*.

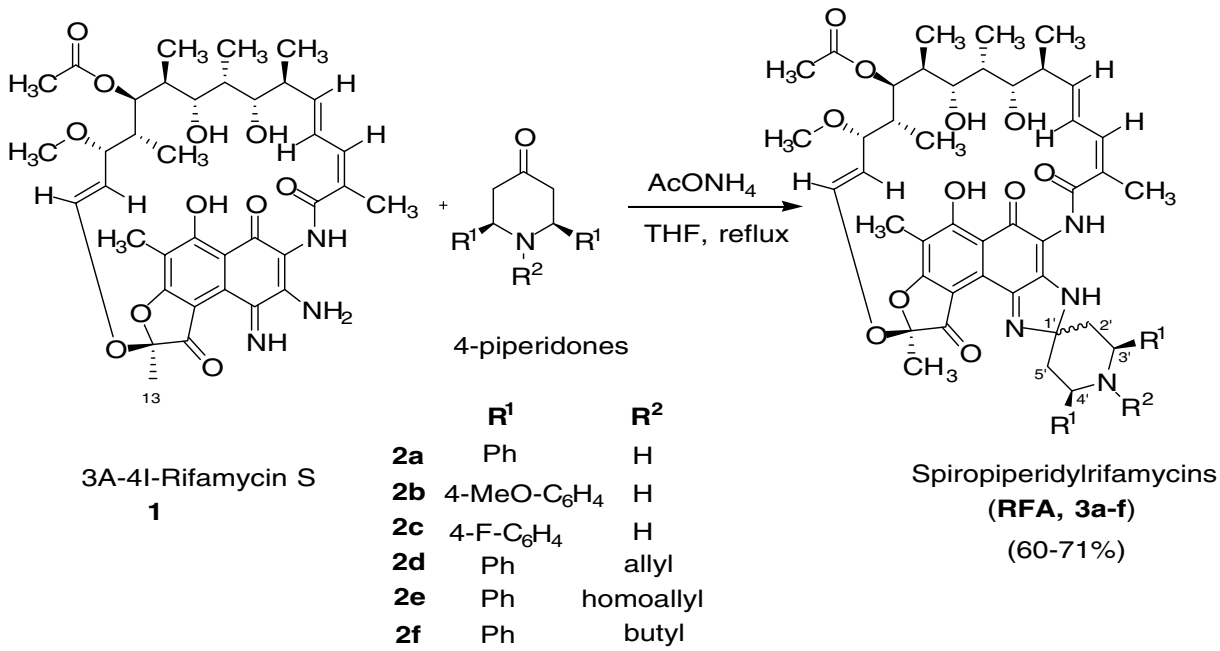
For the preparation of the new spiropiperidylrifamycin derivatives we used a modification of the method previously published for rifabutin and its analogs[11b] involving the condensation reaction between the common intermediate 3-amino-4-iminorifamycin S[19] and several *N*-substituted-4-piperidones (Scheme 1). As far as we are aware, there have not been any examples using 4-piperidones substituted in other positions on the piperidyl moiety. For this reason the first objective of this study was to synthesize a variety of di- and trisubstituted 4-piperidones (**2a**–**f**) to evaluate the importance of the substituents in the biological activity of the new derivatives.

For several years, we have been engaged in the study of the stereoselective synthesis of 4-piperidones by the imino-Diels–Alder reaction of 2-amino-1,3-butadienes with imines.[20] We decided to utilize this methodology toward the preparation of new rifabutin analogs for further biological evaluation. For our studies we chose a variety of *meso*-2,6-disubstituted-4-piperidones (**2a**–**f**)[21] (Scheme 2). The selection of this particular system was motivated by various reasons: symmetrical 4-piperidones would afford two diastereoisomers as reaction products, due to the formation of a new stereocenter at the spiranic carbon atom C-1′, and also would facilitate the structural characterization of these new analogs. Moreover, the new analogs prepared in this way will constitute the first examples in which *meso*-2,6-disubstituted-4-piperidones are used for the condensation reaction with 3-amino-4-iminorifamycin S (**1**).

The condensation reaction of **1** was carried out with the *meso*-2,6-disubstituted 4-piperidones (**2a**–**f**), as shown in



Scheme 1. Retrosynthetic pathway for the synthesis of rifabutin analogs.



	R^1	R^2
2a	Ph	H
2b	4-MeO-C_6H_4	H
2c	4-F-C_6H_4	H
2d	Ph	allyl
2e	Ph	homoallyl
2f	Ph	butyl

Scheme 2. Synthetic pathway for rifabutin analogs **RFA-3**.

Scheme 1. The optimal reaction conditions were achieved using 1 molar equivalent of the intermediate **1** and two equivalents of ammonium acetate in refluxing THF. The progress of the reaction was monitorized by TLC or HPLC until all of the starting material was consumed (1.5–5 h). The reaction mixture was diluted with ether and the organic layer was washed with a buffered solution of $(NH_4)H_2PO_4$ (pH 7.6), dried and evaporated to afford a red solid. The reaction is general regarding the structure of the aryl substituent at the piperidyl ring, from electron rich (*p*-MeO–C_6H_4, **2b**) to electron poor (*p*-F–C_6H_4, **2c**), and the *N*-substituent of the 4-piperidones (H, **2a–c** or alkyl-substituted, **2d–f**). The HPLC analysis of the crude product showed the presence of two diastereoisomeric spiropiperidylrifamycins (**RFA-3a–f**) as the only products in good yield (60–71%) and similar diastereoisomeric ratios (around 3:1) (Table 1). These two diastereoisomers, **M** (major) and **m** (minor), were separated by preparative TLC.

The structural characterization of the new rifabutin analogs **RFA-3a–f** was achieved using 2D NMR spectroscopic experiments through the analysis of their gHSQC and gHMBC spectra, while the configuration of the new spirocyclic C-1′ carbon center was assigned by studying their gNOESY and ROESY spectra for each diastereoisomer.[22] On the major isomer (Fig. 2, **RFA-3aM**) the proposed disposition of the substituents was supported by gNOESY cross peaks between the amine proton NH-3 and the protons H-2′ax and H-5′ax in the piperidine moiety leading to the conclusion that the conformation in solution of this rifabutin analog is a chair with the large substituents facing up in a equatorial orientation giving rise to a (*r*) configuration

Table 1. New spiropiperidylrifamycins (**RFA**) synthesized from *meso*-4-piperidones

Compound	R^1	R^2	Time (h)	Yield[a] (%)	ds (**M**:**m**)[b]
RFA-3a	Ph	H	5	71	68:32
RFA-3b	4-MeO–C_6H_4	H	3	69	74:26
RFA-3c	4-F–C_6H_4	H	3.5	70	75:25
RFA-3d	Ph	Allyl	1.5	68	73:27
RFA-3e	Ph	Homoallyl	1.5	60	73:27
RFA-3f	Ph	Bu	4	62	75:25

[a] Yield of isolated product as a mixture of diastereoisomers.
[b] Determined by ^{1}H NMR of the crude reaction product.

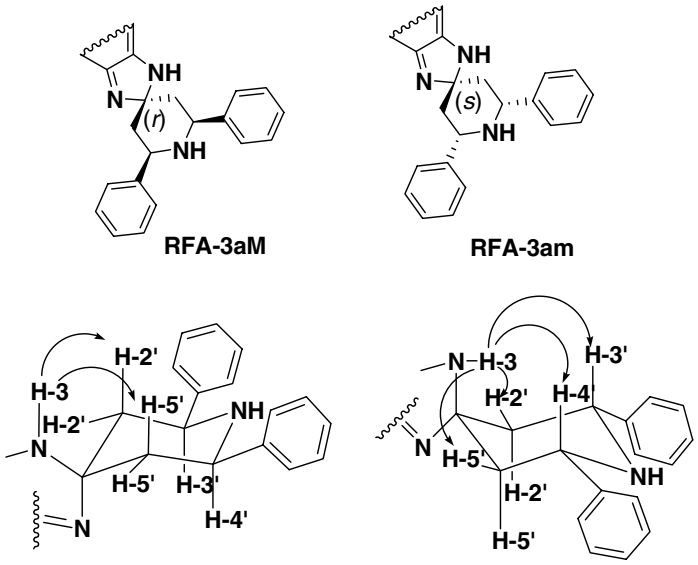


Figure 2. Absolute configuration at the spiranic carbon C-1′ for the new analog **RFA-3a**.

at the spiranic carbon for all the major isomers (**M**). In the minor diastereoisomers (Fig. 2, **RFA-3am**) the gNOESY cross peaks between the amine proton NH-3 and the protons H-3′ax, H-4′ax, H-5′eq, and H-2′eq, corroborate a chair-like conformation in solution for these compounds with the aromatic substituents facing down in an equatorial disposition giving rise to a (*s*) configuration for the C-1′ carbon center in the minor diastereomers (**m**).

To study the structure–antibacterial activity relationships we chose seven of these new rifamycin derivatives: the major isomers of compounds **RFA-3a**–**f** having different groups attached to the nitrogen atom; hydrogen (**RFA-3a**–**cM**) or alkyl (**RFA-3d**–**fM**), and also different aryl substituents at the 2,6 positions of the piperidine ring. Furthermore, we decided to include in this study one of the minor isomers **RFA-3am** to evaluate the effect of the configuration at the spiranic carbon on the biological activity.[23]

MIC values were used to determine the in vitro antimycobacterial activity of these seven new rifabutin analogs synthesized against *M. tuberculosis* (Table 2). Agar dilution susceptibility testing was done according to the proportion method.[24] Together with the target compound, we evaluated susceptibility to the following drugs at the concentrations currently recommended as breakpoints: isoniazid (INH); rifampicin (RMP); streptomycin (SM); ethambutol (EMB) and rifabutin (RFB). In this initial study we included five reference strains of *M. tuberculosis* (MTB): *M. tuberculosis* ATCC 35838 (resistant to RMP and RFB), *M. tuberculosis* ATCC 35820 (resistant to SM), *M. tuberculosis* ATCC 35837 (resistant to EMB), *M. tuberculosis* ATCC 35822 (resistant to INH), and *M. tuberculosis* H37 Rv ATCC 27294 (susceptible to all first line drugs) (entries 1–5). Also, 10 reference strains obtained from the Spanish WHO Supranational Reference Laboratory (Dra. Nuria Martín Casabona, Servicio de Microbiología, Hospital Universitario Vall d'Hebron, Barcelona, Spain) have been tested; six strains were susceptible to RMP and RFB (entry 6) and four strains were resistant to RMP and RFB (entry 7).

All the target compounds tested showed antimycobacterial activity (MIC $\leqslant$0.02 μg/mL) similar to RMP and RFB in *M. tuberculosis* reference strains resistant to drugs other than RMP and RFB (10 strains, entries 2–6). With respect to reference strains resistant to RMP and RFB (5 strains, entries 1 and 7) preliminary results were encouraging, surprisingly, two of our seven analogs (**RFA-3aM** and **RFA-3cM**) showed antimycobacterial activity (80% of resistant strains were susceptible to

Table 2. In vitro activities[a] of RMP, RFB, and the new rifamycins against 15 reference strains of *M. tuberculosis*[b]

Entry	*Mycobacerium tuberculosis* strains	MIC (μg/mL)								
		RMP	RFB	RFA 3aM	RFA 3am	RFA 3bM	RFA 3cM	RFA 3dM	RFA 3eM	RFA3fM
1	ATCC 35838 (RMP^R, RFB^R)	>5	>5	0.5	1	1	0.5	>1	>1	1
2	ATCC 35820 (SM^R)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
3	ATCC 35837 (EMB^R)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
4	ATCC 35822 (INH^R)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
5	H37 Rv	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
6	WHO(6 strains) (RMP^S, RFB^S)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
7	WHO (4 strains) (RMP^R, RFB^R)[c]	>5	>5	0.3 (1)	>1	>1	0.3 (1)	>1	>1	>1
				0.5 (2)			0.5 (2)			
				0.7 (1)			1 (1)			

[a] Each MIC assay was run three times, and for entries 2–6, all of the compounds had MIC values less than 0.02 μg/mL (the limit of detection).
[b] Concentrations used for the following drugs (μg/mL): [INH] = 0.2; [SM] = 4; [EMB] = 5.
[c] The number in brackets for entry 7 indicates the number of strains sensitive at the specified MIC value.

Table 3. In vitro activities[a] of RMP, RFB, and the new rifamycins against 9 strains of MOTT[b]

Entry	Strains	*n*° (% susceptibility)								
		RMP	RFB	RFA 3aM	RFA 3am	RFA 3bM	RFA 3cM	RFA 3dM	RFA 3eM	RFA 3fM
1	MAC (5 strains)	0(0)	1(20)	5(100)	5(100)	3(60)	5(100)	2(40)	4(80)	2(40)
2	*M. kansasii* (4 strains)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)	4(100)

[a] Each MIC assay was run three times.
[b] In all cases the breakpoints tested were 1 μg/mL.

concentrations <0.5 μg/mL and 100% to concentrations <1 μg/mL) exceeding the effectiveness of rifampicin and rifabutin which were resistant to concentrations >5 μg/mL.

Furthermore, we evaluated the biological activity of these seven analogs together with RMP and RFB against clinical isolates of *m*ycobacteria *o*ther *t*han *t*uberculosis (MOTT) (Table 3, entries 1–2). We have tested four strains of *M. kansasii* and five strains of *M. avium-intracellulare* complex (MAC), species that are frequently found in patients with HIV infections in which the treatment uses RFB instead RMP because of contraindications with retroviral agents. Again, in this study, we enhanced the in vitro activity of three of the analogs, **RFA-3aM**, **RFA-3am**, and **RFA-3cM**, which were active at low concentration (MIC <1 μg/mL) against the five strains of *M. avium-intracellulare* complex. It needs to be noted that the five strains of MAC are highly resistant to the clinically used drugs RMP and RFB (MIC >1 μg/mL) (entry 1). In the case of *M. kansasii*, all the seven analogs tested showed similar mycobacterial activity to RMP and RFB against the four clinical isolates (MIC ⩽1 μg/mL) (entry 2).

Accordingly with these results, two of the target compounds synthesized, **RFA-3aM** and **RFA-3cM**, are the most active compounds showing a very high biological activity against all the TB strains tested, including those strains resistant to the clinically used drugs. Taking into consideration the structure of these two potent analogs to explain their higher biological activity we can say that the in vitro antibacterial activity of the novel spiropiperidylrifamycin derivatives synthesized is greater for the *N*-unsubstituted piperidyl derivative and is only marginally affected by the aryl substituent R^1 (electron rich or electron poor). The configuration of the spiranic carbon seems not to be relevant for the biological activity.

In summary, tuberculosis (TB) remains one of the primary causes of human death worldwide. Therefore, there is an urgent need for new chemotherapy agents. Rifamycins have transformed the treatment of tuberculosis over the past 25 years. The development of new rifamycins with increased potencies has renewed interest in this class of compounds and in other potential uses.[25] In this paper, six new spiropiperidyl rifamycin derivatives, which incorporate a new stereogenic center in the molecule, have been synthesized as a mixture of two diastereoisomers. The preliminary in vitro results of the biological evaluation demonstrated very high antimycobacterial activity of these compounds not only against *M. tuberculosis* strains but also against MAC and *M. kansasii*, for which the clinical drugs were ineffective. Rifastures **RFA-3aM**, **RFA-3am**, and **RFA-3cM**, which lack *N*-substitution, displayed the most potent activities. Furthermore, the structure–activity relationships required for the biological activity of the new analogs allow for tolerance in the 2,6-diaryl-substitution, as well as the configuration at the spiranic carbon center.

These initial results are very encouraging. As has been noted above, these new spiropiperidylrifamycin derivatives provide additional opportunities for the development of new antibiotics to overcome resistant

Mycobacteria, and the fact that they are very active against MAC strains opens new applications for these compounds. Further studies of the most potent Rifastures from these investigations, **RFA-3aM** and **RFA-3cM**, are currently in progress to assess their potential cytotoxicity toward human cell lines and mode of action.

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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.2006.08.106.

Complete experimental section for the synthesis and MIC determinations; ^{1}H and ^{13}C NMR data (spectra and tables), and results from elemental analysis for all synthesized Rifastures (**RFA-3a–f**).

References and notes

1. (a) Sensi, P.; Margalith, P.; Timbal, M. *Fármaco, Ed. Sc.* **1959**, *14*, 146; (b) Riva, S.; Silvestri, L. G. *Annu. Rev. Microbiol.* **1972**, *26*, 199; (c) Lounis, N.; Rosceigno, G. *Curr. Pharm. Des.* **2004**, *10*, 3229; (d) Floss, H. G.; Yu, T-W. *Chem. Rev.* **2005**, *105*, 621.
2. (a) Prelog, V.; Oppolzer, W. *Helv. Chim. Acta* **1973**, *56*, 2279; (b) Gallo, G. G.; Martinelli, E.; Pagani, V.; Sensi, P. *Tetrahedron* **1974**, *30*, 3093.
3. (a) Bartolucci, C.; Cellai, L.; Di Filippo, P.; Segre, A.; Brufani, M.; Filocamo, L.; Brizzi, V.; Benedetto, A.; Di Caro, A.; Elia, G. *Il Fármaco* **1992**, *47*, 1367; (b) Bartolucci, C.; Cellai, L.; Di Filippo, P.; Ianelli, M.; Segre, A.; Brufani, M.; Filocamo, L.; Bianco, A.; Guiso, M.; Brizzi, V.; Benedetto, A. *Pharm. Pharmacol. Lett.* **1993**, *3*, 1.
4. (a) Umezawa, H.; Mizuno, S.; Yamazaki, H.; Nitta, K. *J. Antibiot.* **1968**, *21*, 234; (b) Sippel, A.; Hartmann, G. *Biochim. Biophys. Acta* **1968**, *157*, 218; (c) Wehrli, W.; Staehelin, M. *Bacteriol. Rev.* **1971**, *35*, 290; (d) Cellai, L.; Heumann, H.; Baer, G.; Werel, W. *Eur. J. Med. Chem.* **1989**, *24*, 105.
5. (a) Wehrli, W.; Knüsel, F.; Schmid, K.; Staehelin, M. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 667; (b) Wehrli, V.; Staehelin, M. *Biochim. Biophys. Acta* **1969**, *182*, 24; (c) Sensi, P. *Pure Appl. Chem.* **1975**, *41*, 15.
6. Sensi, P.; Maggi, N.; Füresz, S.; Maffii, G. *Antimicrob. Agents Chemother.* **1966**, 699–714.
7. Lancini, G.; Zanichelli, W. Structure–Activity Relationship among the Semisynthetic Antibiotics. In Perlmann, D., Ed.; Academic Press: New York, 1977; pp 531–600.
8. Other trivial names used are: rifamycin AMP and rifaldazine.
9. (a) Maggi, N.; Pasqualucci, C. R.; Ballota, R.; Sensi, P. *Chemotherapy* **1966**, *11*, 285; (b) Gallo, G. G.; Radaelli, P. Analytical Profiles of Drug Substances. In Florey, E. K., Ed.; Academia Press: New York, 1976; pp 467–513.
10. Abbreviated name for 3-amino-4-deoxo-4-imino-3,4-imidazolinyl-1H-[2-*spiro*(4- piperidyl)]rifamycin S.
11. (a) Sanfilippo, A.; Della Bruna, C.; Marsili, L.; Morvillo, E.; Pasqualucci, C. R.; Schioppacasi, G.; Ungheri, D. *J. Antibiot.* **1980**, *33*, 1193; (b) Marsili, L.; Pasqualucci, C. R.; Vigevani, A.; Gioia, B.; Schioppacassi, G.; Oronzo, G. *J. Antibiot.* **1981**, *34*, 1033; (c) Medeiros, M.; Costa, M.; Figueiredo, M.; Rosa, M.; Curto, M.; Santos, L.; Crus, M.; Gaspar, M.; Feio, S. WO 2004/005298 A1.
12. (a) Marsili, L.; Rosetti, V.; Pasqualucci, C.R. US Patent 4,086,225, 1978; (b) Marsili, L.; Rossetti, V.; Pasqualucci, C.R. German Patent Appl. 2,825,445, 1979.
13. (a) Marsili, L.; Rosetti, V.; Pasqualucci, C.R. US Patent 4,219,478, 1980; (b) Della Bruna, C.; Schioppacassi, G.; Ungheri, D.; Jabes, D.; Morvillo, E.; Sanfilippo, A. *J. Antibiot.* **1983**, *36*, 1502.
14. (a) Kunin, C. M. *Clin. Infect. Dis.* **1996**, *22*, S3; (b) Chaisson, R. E. *Clin. Infect. Dis.* **1996**, *22*, S61.
15. (a) Ungheri, C. D.; Della Bruna, C.; Sanfilippo, A. *M. Tuberculosis Drugs Exp. Clin. Res.* **1984**, *10*, 681; (b) Ungheri, D.; Della Bruna, C.; Sanfilippo, A. *G. Ital. Chemioterapic* **1984**, *31*, 211.
16. (a) Espinal, M. A.; Kim, S. J.; Suárez, P. F.; Kam, K. M.; Khomenko, A. G.; Migliori, G. B., et al. *JAMA* **2000**, *283*, 2537; (b) Gardner, C. A.; Acharya, T.; Pablos-Méndez, A. *Clin. Chest. Med.* **2005**, *26*, 341.
17. Rieder, H.L. Intervetions for Tuberculosis Control. International Union against Tuberculosis and Lung Disease, Paris, France, 2002.
18. Spanish Patent ES 2 246 155 A1, 2005. International Patent PCT WO 2006/027397 A1.
19. (a) Obtained from Rifamycin S according to different procedures: Marsili, L.; Rosseti, V.; Pasqualucci, C. US Patent 4,017,481, 1977; (b) Rossetti, V.; Marsili, L. D.E. Patent 2847427, 1979; (c) Marchi, E.; Montecchi, L. G.B. Patent 2009744, 1979.
20. Barluenga, J.; Aznar, F.; Valdés, C.; Cabal, M.-P. *J. Org. Chem.* **1993**, *58*, 3391.
21. García, A-B.; Valdés, C.; Cabal, M-P. *Tetrahedron Lett.* **2004**, *45*, 4357.
22. Rubio, E.; Merino, I.; García, A-B.; Cabal, M.-P.; Ribas, C.; Bayod-Jasanada, M. *Magn. Reson. Chem.* **2005**, *43*, 269.
23. The results of this experiment showed that the configuration at the spiranic carbon does not improve biological activity against the susceptible strains (entries 2–6, Table 2), and that bioactivity is diminished considerably against the resistant TB strain (entries 1 and 7, Table 2). Based on these findings, we do not feel that it would be warranted to check the activity of the other five minor isomers.
24. Canetti, G.; Rist, N.; Grosset, J. *Rev. Tuberc. Pneumol.* **1963**, *27*, 217.
25. Rothstein, D. M.; Hartman, A. D.; Cynamon, M. H.; Eisenstein, B. H. *Expert Opin. Investig. Drugs* **2003**, *12*, 255.