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Spin-labeled rifamycin: biological activity

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3-[(2,2,6,6-Tetramethylpiperidine-4-ylimino)methyl]rifamycin (**4**) and spin-labeled rifamycin-3-[(2,2,6,6-tetramethyl-1-oxyl-piperidine-4-ylimino)methyl]rifamycin (**1**) were prepared. The structures of these compounds were determined by IR, UV, MS and ^{1}H NMR of **4**. The ESR-spectrum of **1** is a symmetric triplet signal, characteristic of nitroxyl radicals, g = 2.0025. An *in vitro* comparative study of the cytotoxicity and antitumor activity of **1**, **4** and the initial 3-formyl-rifamycin was carried out in concentrations from 0.1 to 0.001 mM on cells before and after oxidative stress (preliminary irradiation 7Gy) on MH3924A-hepatoma rat cells, 293 transformed human fibroblasts, NBK transformed human fibroblasts and HT 1080 human fibrosarcoma. The compounds showed a cytostatic effect to 85%, with **1** being less toxic in the hepatoma cell line. In human melanoma cell lines **1** showed a higher toxicity than **4**. All the derivatives (**1** and **4**) have *in vitro* antibacterial activity comparable with that of rifampicin.

1. Introduction

Chemotherapy of tuberculosis is indicated in the case of disease, as well as in latent tuberculosis infection. Standard medication for drug-susceptible tuberculosis consists of isoniazid and rifampicin for the first two months with additional pyrazinamide and ethambutol treatment. Attention must be paid to the side effects of antituberculosis medications.

Rifampicin is an antibiotic with a broad spectrum of antibacterial activity against gram(+) and gram(−) bacteria including *Mycobacterium tuberculosis* (Greinert and Zabel 2003), *Staphylococcus aureus* and *Mycobacterium leprae* (Dhople and Namba 2003; Davies and Yew 2003).

The mechanism of action of rifamycin and its derivatives has been investigated (Magnarin et al. 2004; Giraldi and Decorti 2004; Ferguson et al. 2001; Steffek et al. 2003), as well as the relationship between the chemical structure and the action of these products. It was found that during the metabolism of the latter, oxygen radicals are formed and some authors attribute the high hepatotoxicity of rifamycin and its derivatives to these radicals (Kukielka and Cederbaum 1992; Rao and Cederbaum 1997). Using diphenylpicryl hydrazil, it was found that some derivatives of rifamycin possess radical-scavenging properties (Karunakar et al. 2003). The high general toxicity of rifamycin (Apseloff 2003) has led to the synthesis of many new derivatives, by introducing different active groups such as hydrazine (Marsili et al. 1982), semicarbazide and many others into the rifamycin molecule. The possibilities of combined action with other preparations (Greinert and Zabel 2003) have been investigated, as well as incorporation into liposomes (Vyas et al. 2004) and nanoparticles (Skidan et al. 2003).

It is known that the inclusion of a nitroxyl radical, i.e. a radical-scavenger, into the molecules of bioactive compounds (spin-labeled compounds) leads to a decrease of their general toxicity, without changing their activity. The presence of a free electron in these compounds makes them free radicals or spin-labeled compounds. Examples are the spin-labeled compounds obtained in the series of N-mustards (Raikova 1977; Raikova et al. 1982), aziridines (Sosnovsky 1990), nitrosoureas (Raikov et al. 1985; Raikov et al. 1990), triazenes (Raikov et al. 1993) and some antibiotics (Hsia and Piette 1979; Emanuel et al. 1985).

These facts prompted us to synthesize a new spin-labeled rifamycin – 3-[(2,2,6,6-tetramethyl-1-oxyl-piperidine-4-ylimino)methyl]rifamycin (**1**) through direct interaction of 3-formyl-rifamycin (**2**) with 4-amino-tetramethylpiperidine-1-oxyl (**3**).

In the present study, we describe the results of the synthesis and some properties of **1** and 3-[(2,2,6,6-tetramethylpiperidine-4-ylimino)methyl]rifamycin (**4**). An *in vitro* comparative study of the cytotoxic effects of the newly synthesized derivatives in different types of transformed and tumor cells was also carried out. The antimicrobial activity of the compounds is also described.

2. Investigations and results

2.1. Synthesis of the compounds

The route of synthesis and structures of the spin-labeled rifamycin (SLR) **1** and its amine **4** are shown in Scheme 1.

UV-VIS data in methanol and IR data in KBr of the compounds are given in Tables 1 and 2.

The IR-spectrum of **1** shows characteristic band at 1399 cm^{-1} for $N^{\bullet}-O$ groups.

The ESR-spectrum of **1** has a symmetrical triplet signal, similar to that of the nitroxyl radicals g = 2.0025 (Fig. 1).

Scheme 1

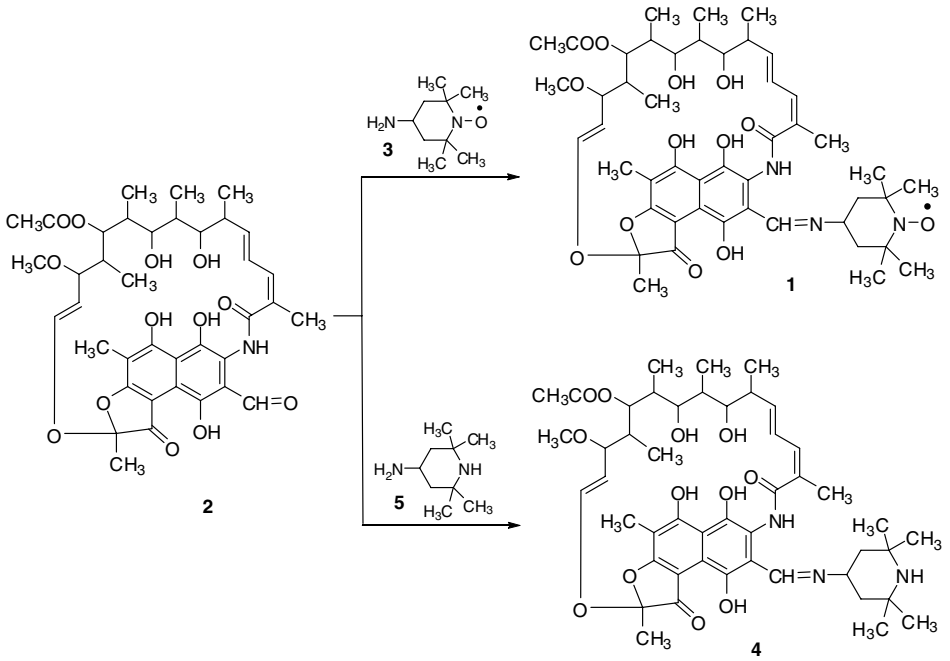


Table 1: UV-VIS data for compounds in methanol

Compd.	Nm	ε (l/Mcm)	nm	ε (l/Mcm)	nm	ε (l/Mcm)	nm	ε (l/Mcm)
2	235	17340	–	–	330	8375	490	5675
4	240	34800	–	–	320	11090	490	8696
1	230	34960	280	22795	358	11100	510	4845

Table 2: IR data (cm^{-1}) for compounds in KBr

	Frequence		
	4	**1**	Lit. Data*
ν –O–H	3587–3167	3735–3180	3500–3000
ν $-CH_2,-CH_3$	2970–2880	3125–2880	2930
ν –C=O	1715, 1701, 1682	1718, 1680	1730, 1715
ν –C=N	1652	1654	1670
ν –C=C–	1575–1540	1570–1540	1570
ν –C–N	1470–1324	1458–1300	1400
ν –C–O–C	1260–1018	1260–1019	1255–1020
ν –N–O	1399		

* (Galo and Radaell 1974)

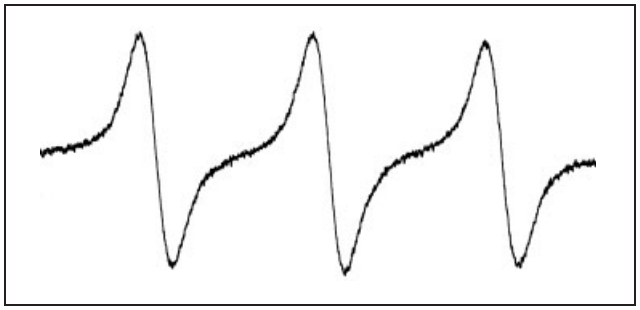


Fig. 1: ESR-spectrum of **1** in methanol at 80 K, mod. 2.5 G, t.c. 100 μs, sc.t. 200 s

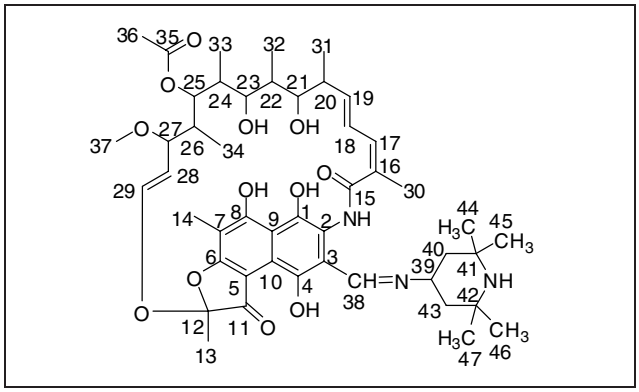


Fig. 2: USAN numbering of compound **4**

Table 3: ^{1}H NMR-data for 4 in $CDCl_3$

H-atom	δ (m)	J (Hz)	δ (ppm)*
CH= (38)	8.14 s		8.22
CH_3 (13)	1.80 s		1.82
CH_3 (14)	2.28 s		2.23
H (17,18)	6.2; 6.7 m	10–12	6.3, 6.8
H (19)	5.94 dd	5–11	5.92
H (20)	2.27 ddq	5–10	2.26
H (21)	3.70 dd	2–7	3.78
OH (21, 23)	3.15; 4.5 bs		3.2, 4.2
H (22)	1.73 ddq	5–10	1.70
H (23)	3.07 dd	5–12	3.04
H (24)	1.55 ddq	3–7	1.52
H (25)	4.98 dd	5–8	4.96
H (26)	1.23 ddq	2–6	1.22
H (27)	3.57 dd	5–7	3.58
H (28)	4.92 dd	5–9	5.00
H (29)	6.22 d	9–12	6.20
CH_3 (30)	1.96 s		2.10
CH_3 (31)	0.89 d	7	0.88
CH_3 (32)	1.00 d	10	1.01
CH_3 (36)	2.00 s		2.06
CH_3 (37)	3.23 s		3.05
CH_3 (44–47)	1.24 m	2–5	–
CH_2 (40, 43)	2.8–3.2 m	3–5	2.9–3.3

* (Galo and Radaell 1974)

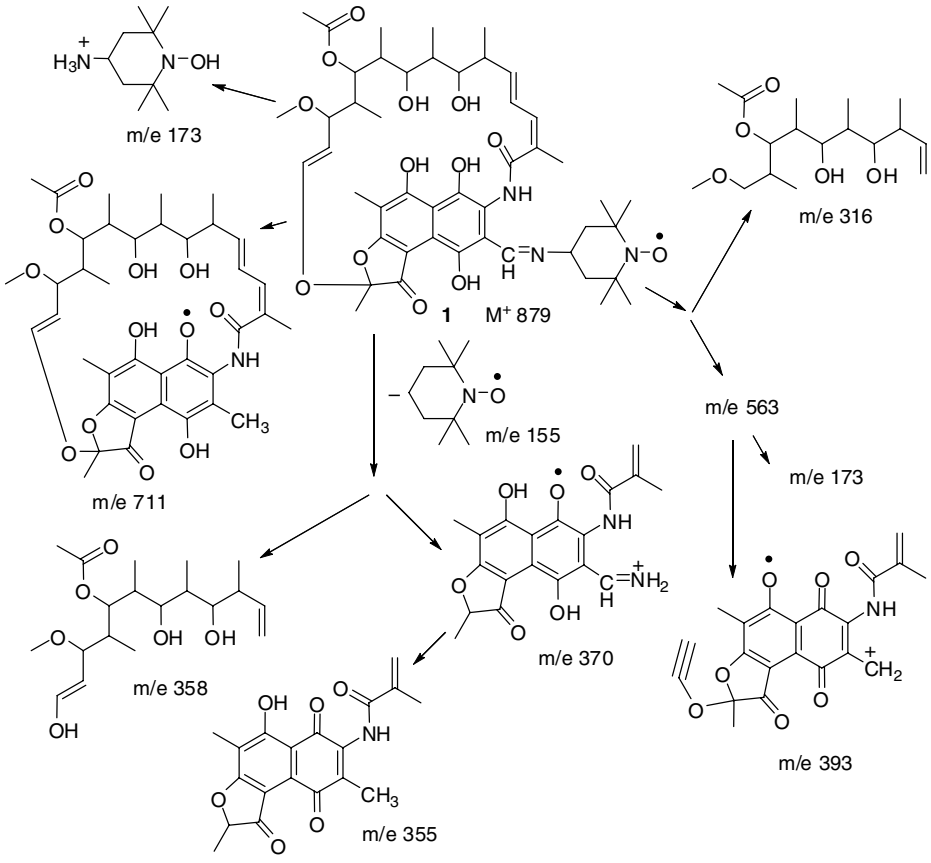
The results of the ^{1}H NMR spectrum of compound **4** are shown according to USAN numbering (Fig. 2) and are tabulated in Table 3.

The major fragments in the MS spectra of **1** and **4** are represented in Schemes 2 and 3.

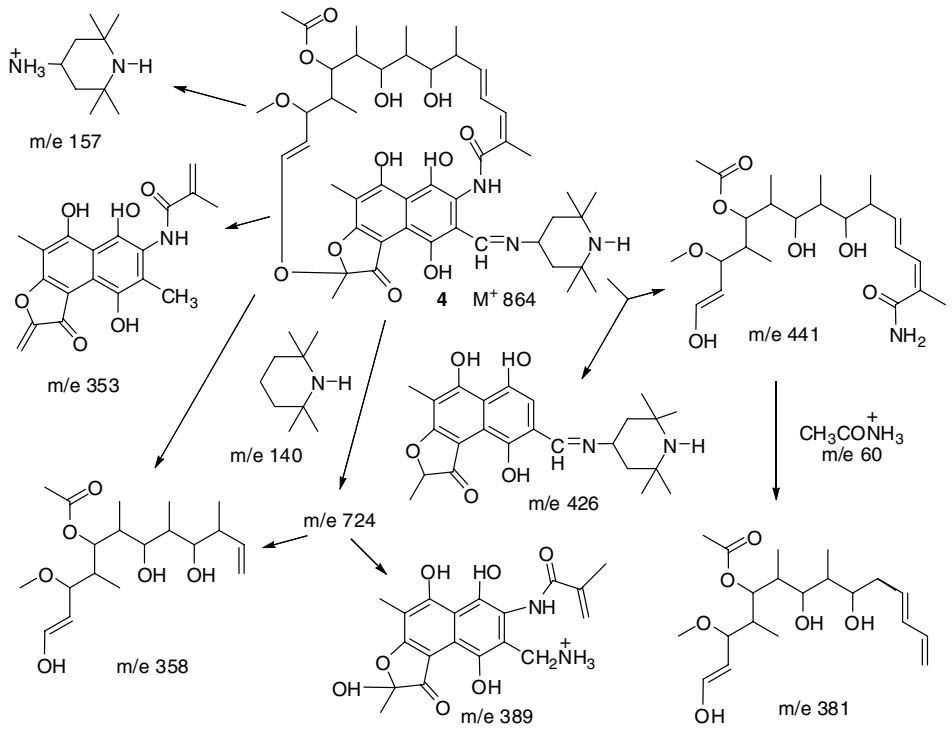
2.2. Cytotoxicity of compounds 1, 4 and 2

Compounds **2** and **1** showed no differences in their cytotoxic effects on human transformed cells (NBK, MH293T, HT1080) either under natural culture conditions or in the

Scheme 2



Scheme 3



case of oxidative stress. However, the spin-labeled derivative of rifamycin had a two fold reduction in toxicity on the rat hepatoma cell line. Cytotoxicity of **1**, **4** and **2** before oxidative stress is shown in Fig. 3. The cells were treated with the indicated concentrations of the respective compounds and cell survival was measured 3 days later by MTT. Calculations are based on taking the same amount of DMSO without substance added as 100%.

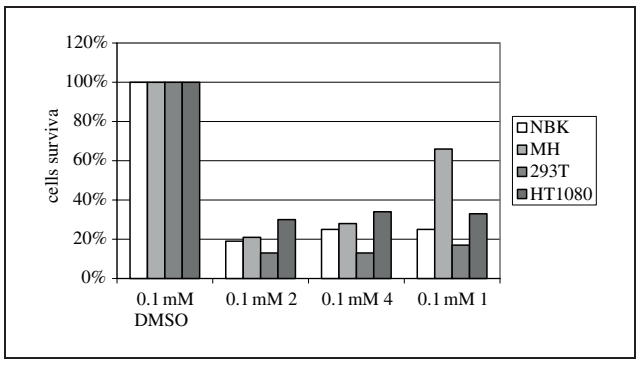


Fig. 3: Cytotoxicity of **1**, **2** and **4** before oxidative stress

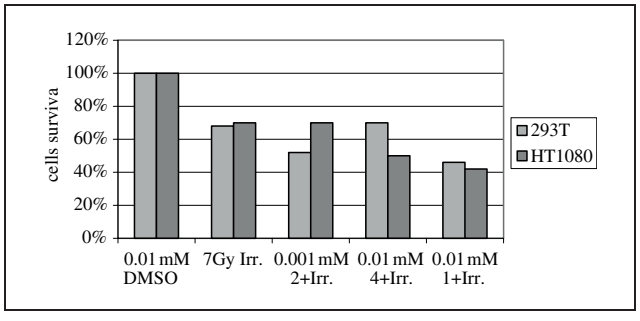


Fig. 4: Cytotoxicity of **1**, **4** and **2** after oxidative stress (7 Gy irradiation)

Cytotoxicity of **1**, **2** and **4** after oxidative stress (7 Gy irradiation) is shown in Fig. 4. The cell survival was measured in the same way as in Figure 3 but the cells were irradiated as indicated prior to the application of the respective compounds.

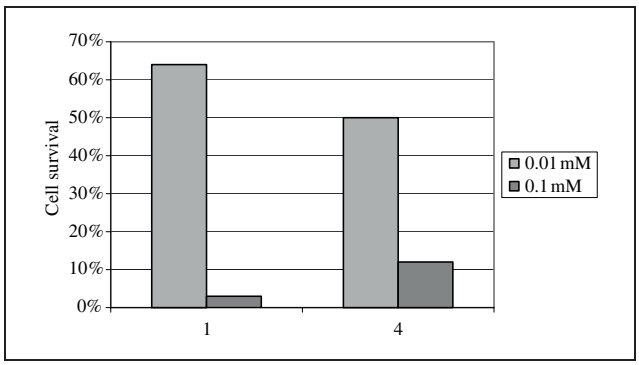


Fig. 5: Human melanoma cell lines (Carney) treated with **1** and **4**

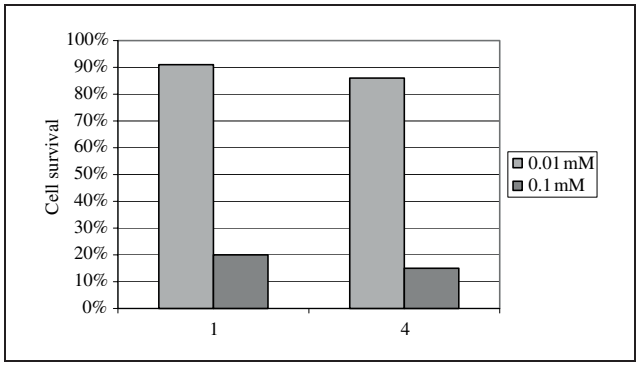


Fig. 6: Human melanoma cell lines (Aderhold) treated with **1** and **4**

Table 4: *In vitro* antibacterial activity of representative rifamycin derivatives

Compd.	MIC mg/ml							
	S. aureus tour L165	*Strept. faecalis*	*Strept. pyogenes*	*Pseudomonas aerug.*	*Escherchia coli*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Mycobacterium tuberculosis $H_{37}Rv$*
1	0.048	0.36	0.28	10	>10	10	10	0.01
4	0.063	0.51	0.41	>20	>10	10	20	0.02
2	0.085	0.73	0.53	>20	>10	>20	>20	0.04
Rifampicin	0.008	0.28	0.31	5	5	2.7	10	0.01

Compounds **1** and **4** were used for treating the Carney and Aderhold human melanoma cell lines at concentrations of 0.01 and 0.1 mM. Results are shown in Figs. 5 and 6.

2.3. Microbiological activity

Derivatives **1** and **4** have *in vitro* antibacterial activity comparable with that of rifampicin. The *in vitro* antibacterial activities of the compounds **1** and **4** against several Gram-positive and Gram-negative bacterial species was compared with those of **2** and rifampicin. Against *Mycobacterium tuberculosis*, **1** and **4** were more active than the comparators **2** (initial rifamycin) and rifampicin (Table 4).

Compound **1** may be noted among the three compounds tested as having the highest antimicrobial activity. It surpassed **2** and **4** in the tests with each of the screened microbial species. Compound **1** displays activity identical to that of rifampicin as regards *Mycobacterium tuberculosis*, *Proteus vulgaris* and both Streptococci (*Streptococcus faecalis* and *Streptococcus pyogenes*).

2.4. Toxicity

Groups of five male and five female mice received a single i.p. dose of compound **1** suspended in 0.5% Methocel K15 Premium (Dow Chemical). The LD_{50} with 95% confidence limits was calculated from the mortality data using probit analysis. In the mouse, the LD_{50} of compound **1** was 361.1 mg/kg, compared with rifampicin 287.7 mg/kg; spin labeled rifamycin showed a lower toxicity than rifampicin.

3. Discussion

Numerous studies on spin labeled (SL) compounds show that their properties differ to a great extent from those of their initial analogues. For example, many SL-antitumor compounds have been proved to possess a high antitumor effect, broad spectrum of anticancer activity and relatively low general toxicity in comparison to that of their diamagnetic analogues. Spin-labeled derivatives of the anthracyclinic antibiotics, actinomycin (Hsia and Piette 1979) and rubomycin (Emanuel et al. 1985) have also been synthesized.

The free nitroxyl radical, present in the rubomycin molecule, significantly decreases the cardiotoxicity of this compound and increases its antitumor activity. It is worth mentioning that spin-labeled rubomycin has a different spectrum of anticancer activity, as compared to its unlabeled (diamagnetic) analogue. Spin-labeled rubomycin suppresses the growth of melanoma B_{16} and adenocarcinoma 755, while solid transplantation tumors seem not to be sensitive to rubomycin. In this case, stable free radicals in the rubomycin molecule probably serve as radical scavengers towards unstable toxic radicals, formed during the metabolism of this drug (Emanuel et al. 1985). Furthermore, spin-labeled compounds, such as tetracycline and others, also acquire antimelanoma activity (Pezesch and Pezesch 1992). It has been shown that ^{14}C rifamycin accumulates in melanin-containing tissues (Boman 1975).

The penetration of rifamycin and isoniazid derivatives in liposome membranes has been studied by ^{1}H NMR and it was found that isoniazid is located on the membrane surface, while rifamycin penetrates deeply into them (Boman 1975).

By means of ESR, similar studies could also be carried out with **1**, and in that way its penetration through membranes, such as those of tuberculosis and other bacteria, can be investigated. In view of the special features of these waxy and relatively hard to penetrate membranes, **1** can provide highly valuable data on their properties.

Our studies showed that **1** possesses an antitumour activity with respect to fibrosarcoma, which has been previously shown in investigations with spin-labeled derivatives of other substances (Gnewuch and Sosnovsky 1997). The lower toxicity of **1** to hepatoma cells deserves further studies in normal human hepatocytes as well as *in vivo*, in order to establish whether this rifamycin derivative has a reduced hepatotoxicity. As expected from the above data, the spin-labeling of rifamycin also seems to improve its antimelanoma activity.

These first results with **1** will inspire future investigations of the antitumour effect of the compound on a wider spectrum of tumour cells in different concentrations and conditions. Last but not least, the presence of a nitroxyl radical will provide the opportunity to use it in the field of magnetic resonance imaging (MRI) for pharmacokinetic studies.

4. Experimental

4.1. Reagents and equipment

4-Amino-2,2,6,6-tetramethyl-piperidine (**5**) and the other reagents used were of high purity grade from Sigma Aldrich. **4** and **1** were obtained by direct synthesis.

All the UV-VIS spectra were recorded on Specord UV-VIS (Germany) and Unicam SP-800 spectrophotometers in methanol solution with concentration 4.10^{-5} mol/l and d 0.5 cm. A BOMEM-Michelson 100 FTIR spectrophotometer was used to conduct all the IR spectroscopy experiments. The IR spectra were obtained using KBr tablets of the compounds. The NMR spectra of **4** were produced using a 400 AMX Bruker spectrometer in d_6 DMSO solution. MS E:/TSQ-DATA were used to determine the molecular weight of each drug sample. The results were interpreted and reported as the relative abundance of fragments.

4.2. Synthesis of the compounds

4.2.1. 3-[(2,2,6,6-Tetramethylpiperidine-4-ylimino)methyl]rifamycin (**4**)

0.73 g (1 mM) of 3-formyl-rifamycin were dissolved in 30 ml ethyl acetate. To this solution, 0.16 g (1 mM) of **5** in 10 ml ethyl acetate were added at room temperature with continuous stirring for 2.5 h. Upon cooling hexane was added and the solution was cooled to −10 °C for 24 h. The precipitate obtained was collected by filtration as an orange-red powder (0.76 g, 89.1%) m.p. 171–172 °C. TLC $R_f = 0.43$ (silica gel, $CHCl_3/CH_3OH$ (8 : 2)). $C_{47}H_{65}N_3O_{12}$

4.2.2. 3-[(2,2,6,6-Tetramethyl-1-oxyl-piperidine-4-ylimino)methyl]rifamycin (**1**)

0.73 g (1 mM) of 3-formyl-rifamycin were dissolved in 20 ml butyl acetate/hexane (1 : 1). To this solution, 0.2 g (1.2 mM) of **3** in 10 ml butyl acet-

ate were added at room temperature with continuous stirring for 2 h. The color of the solution changed from red to deep violet. Upon cooling hexane was added and the solution was cooled to −20 °C for 24 h. The precipitate obtained was collected by filtration as a violet powder (0.73 g, 82.9%). TLC $R_f = 0.37$ (silica gel, $CHCl_3/CH_3OH$ (8 : 2)).
The crude product was dissolved in 5 ml of a mixture of $CHCl_3/CH_3OH$ (8 : 2) and subjected to flash (column) chromatography on silica gel, which was eluted with $CHCl_3/CH_3OH$ (8 : 2). After evaporation under vacuum at 25 °C deep violet crystals of **1** were optained.
$C_{47}H_{64}N_3O_{13}$

4.3. Cell strains used

MH3924A-hepatoma rat cells, 293 transformed human fibroblasts, NBK transformed human fibroblasts and HT 1080 human fibrosarcoma were obtained from ATCC. All cell lines were cultured in RPMI medium (MH3924A, referred to in the text as MH) or DMEM (MBK, MH293T, HT1080) supplemented with 10% Fetal Calf Serum.

4.4. Gamma-irradiation

Gamma-irradiation of 7 Gy was performed in a Gammacell 1000 gamma irradiator for the induction of oxidative stress.

4.5. Cytotoxicity assay

An MTT (3,4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay in a 96 well plate was performed to test the viability of the cells. Briefly, 20 µl of a 5 mg/ml MTT solution in PBS were added to each well. After 3 h incubation of the cells with MTT at 37 °C, the culture medium was aspirated and the plates were dried for 15 min. Isopropanol was added to dissolve the colored reaction product, and the optical density was measured using an ELISA reader at 570 nm. For cell culture treatment **2**, **4** and **1** were dissolved in DMSO and adjusted to the indicated (see legends to Figures) final concentrations in the culture medium.

4.6. Microbiological activity

Minimal inhibitory concentration (MIC) was determined by the serial twofold dilution technique in Difco Antibiotic medium No3 with 15‰ of Difco Agar for Gram-positive and Gram-negative bacteria and Difco Bacto-Dubos Albumin Broth for *Mycobacterium tuberculosis* $H_{37}Rv$. The MICs were the lowest concentrations of the antibiotic which prevented any visible growth after 1 day (*M. tuberculosis*) incubated at 37 °C for 7 days.
Other species were incubated at 37 °C for 18–24 h.

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