Rifamycins.[1] XXXV.[2] Amides and Hydrazides of Rifamycin B[3]

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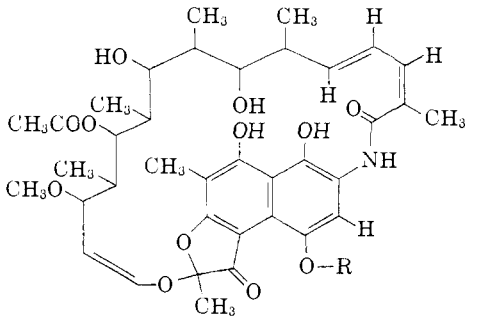
It is known that by splitting off the glycolic acid moiety from the molecule of rifamycin B the antimicrobial activity increases dramatically in rifamycin S and rifamycin SV. Starting from the hypothesis that the presence of a free carboxyl group in the molecule of rifamycin B might forbid the display of the activity, a series of amides and hydrazides of rifamycin B has been prepared by allowing rifamycin B to react with amines or hydrazines in the presence of dicyclohexylcarbodiimide as dehydrating agent. Analytical data and antibacterial activities of 49 amides and 26 hydrazides are reported. Among them, the N,N-disubstituted rifamycinamides and the N,N,N'-trisubstituted rifamycinhydrazides appear to possess considerable antibacterial activity against gram-positive bacteria and *Mycobacterium tuberculosis*, of the same order of rifamycin SV. The *in vivo* activity of some rifamycinamides and rifamycinhydrazides in experimental staphylococcal infection in mice is very high and in some cases higher than that of rifamycin SV. The acute toxicity of a series of derivatives of rifamycin B is also reported.

Rifamycin B is a substance isolated from the fermentation broths of *Streptomyces mediterranei*.[4] It has been already reported that rifamycin B can be converted into rifamycin SV, through rifamycin O and rifamycin S.[5] Rifamycin SV shows considerable antibacterial activity and is now employed widely in the treatment of staphylococcal infections, of infections of the biliary tract, and of tuberculous and leprous infections.[6] Considering the structural relations between rifamycin B and rifamycin SV,[7] we have hypothesized that the low activity of rifamycin B could be due to the presence of a free carboxyl group in its molecule. Therefore, derivatives of rifamycin B with the carboxyl group blocked by conversion into amides, hydrazides, or esters might show a high antibacterial activity.



rifamycin B, R = CH_3COOH
rifamycin SV, R = H

The present paper deals with the preparation and properties of a large number of amides and hydrazides of rifamycin B. The greater part of the amines necessary for the preparation of rifamycinamides were obtained from commercial sources; only a few of them were prepared according to literature data. The N,N,N'-trialkylhydrazines were prepared by treating the N,N-dialkylhydrazines with formaldehyde to give the corresponding N,N-dialkyl-N'-methylenhydrazines.[8] These compounds were either reduced to the N,N-dialkyl-N'-methylhydrazines or treated with alkylmagnesium bromide to give the N,N-dialkyl-N'-alkylhydrazines.[9] The process for preparing the amides or hydrazides of rifamycin B consisted in treating rifamycin B with amines or hydrazines in the presence of dehydrating agents such as dicyclohexylcarbodiimide.[10]

Amides and hydrazides of rifamycin B are orange-yellow substances, very soluble in methanol, ethanol, benzene, acetone, ethyl acetate, and scarcely soluble in water. They show an acidic function (pK 3.0–4.6), which is to be attributed to the acidic phenolic group in the *peri* position, and therefore they form neutral salts with organic and inorganic bases. The solubility in water of the sodium salts of the amides and hydrazides of rifamycin B is generally rather good. The sodium salts of hydrazides are more soluble in water than those of the amides. Among the amides the less soluble are those bearing either aryl or long aliphatic chains. The amides and hydrazides of rifamycin B show in the ultraviolet and in the visible region of the spectrum absorption maxima similar to those of rifamycin B [220–225 mμ (ϵ ~40,000–44,000), 304–306 (~20,000–22,000), and 425 (15,000–16,500)]. The melting points of these derivatives of rifamycin

(1) The name rifamycin has been adopted instead of rifomycin as in the first papers in order to differentiate the name more fully from the commercial names of other antibiotics.

(2) Paper XXXIV: J. E. Thiemann, C. Hengeller, A. Virgilio, O. Buelli, and C. Icciardello, *Appl. Microbiol.*, **12**, 269 (1964).

(3) Presented in part at the IIIrd International Congress of Chemotherapy, Stuttgart, July 22–27, 1963.

(4) (a) P. Sensi, P. Margalith, and M. T. Timbal, *Farmaco* (Pavia), *Ed. Sci.*, **14**, 146 (1959); (b) P. Sensi, A. M. Greco, and R. Ballotta, *Antibiot. Chemotherapy*, 262 (1960); (c) P. Margalith and G. Beretta, *Mycopathol. Mycol. Appl.*, **13**, 21 (1960); (d) P. Margalith and B. Pagani, *Appl. Microbiol.*, **9**, 325 (1961).

(5) (a) P. Sensi, M. T. Timbal, and G. Maffii, *Experientia*, **16**, 412 (1960); (b) P. Sensi, R. Ballotta, A. M. Greco, and G. Gallo, *Farmaco* (Pavia), *Ed. Sci.*, **16**, 165 (1961).

(6) The literature on the clinical applications of rifamycin SV (Rifocin®) is listed in a review by P. Sensi, "Progress in Organic, Biological and Pharmaceutical Chemistry," Societa Editoriale Farmaceutica, Milano, 1964, pp. 337–421.

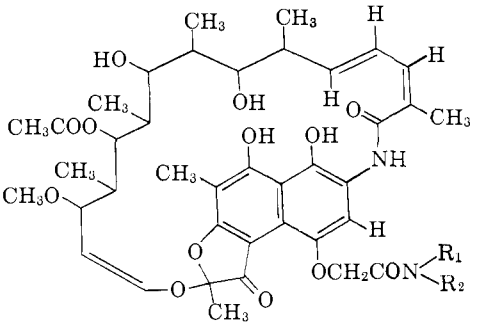
(7) (a) V. Prelog, Proceedings of the Symposium on Chemistry and Biochemistry of Fungi and Yeasts, 551 (1963); (b) V. Prelog, *Chemotherapia* (Basel), **7**, 133 (1963); (c) W. Oppolzer, V. Prelog, and P. Sensi, *Experientia*, **20**, 336 (1964); (d) M. Brufani, W. Fedeli, G. Giacomello, and A. Vaciago, *ibid.*, **20**, 339 (1964); (e) J. Leitich, W. Oppolzer, and V. Prelog, *ibid.*, **20**, 343 (1964).

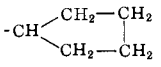
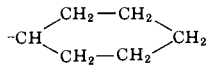
(8) I. B. Class, I. G. Astore, and T. S. Oakwood, *J. Am. Chem. Soc.*, **75**, 2937 (1953).

(9) B. V. Joffe and L. E. Porosekin, *Zh. Obshch. Khim.*, **29**, 3154 (1959).

(10) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

TABLE I
RIFAMYCIN AMIDES



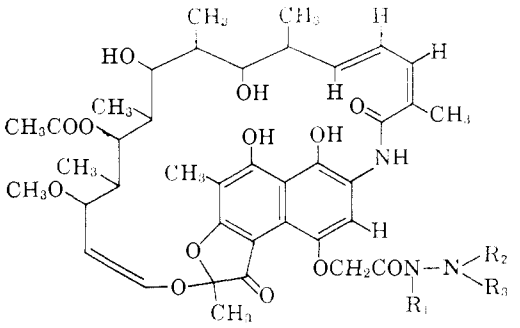
Compd.[a]	R_1	R_2	Yield, %[b]	Formula	Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N
1	H	H	45	$C_{39}H_{50}N_2O_{13}$	62.05	6.68	3.71	61.88	7.36	3.92
2	H	CH_3	39	$C_{40}H_{52}N_2O_{13}$	62.48	6.82	3.64	62.50	6.99	3.94
3	H	C_2H_5	70	$C_{41}H_{54}N_2O_{13}$	62.90	6.95	3.58	62.38	7.33	3.73
4	H	C_3H_7	85	$C_{42}H_{56}N_2O_{13}$	63.30	7.08	3.51	62.95	7.52	3.80
5	H	i-C_3H_7	92.5	$C_{42}H_{56}N_2O_{13}$	63.30	7.08	3.51	62.85	7.60	3.76
6	H	t-C_4H_9	67	$C_{43}H_{58}N_2O_{13}$	63.68	7.21	3.45	63.20	7.54	3.46
7	H	DL-$CH_3CH(CH_2)_4CH_3$	95	$C_{46}H_{64}N_2O_{13}$	64.77	7.56	3.28	65.01	8.04	3.06
8	H	C_6H_5	40	$C_{45}H_{54}N_2O_{13}$	65.21	6.52	3.35	64.70	6.88	3.33
9	H	C_6H_4Cl-p	91	$C_{45}H_{53}ClN_2O_{13}$	62.45	6.17	3.24 (Cl, 4.10)	61.82	6.31	3.11 (Cl, 4.61)
10	H	C_6H_4Br-p	98	$C_{45}H_{53}BrN_2O_{13}$	59.40	5.87	3.08 (Br, 8.78)	59.25	6.26	3.14 (Br, 8.86)
11	H	C_6H_4I-p	99	$C_{45}H_{53}IN_2O_{13}$	56.48	5.58	2.93 (I, 13.26)	56.37	5.30	2.78 (I, 13.55)
12	H	$CH(OH)CH_3$	75	$C_{41}H_{54}N_2O_{14}$	61.64	6.81	3.51	61.60	6.91	3.79
13	CH_3	CH_3	20	$C_{41}H_{54}N_2O_{13}$	62.90	6.95	3.58	63.00	7.47	3.58
14	C_2H_5	C_2H_5	93	$C_{43}H_{58}N_2O_{13}$	63.68	7.21	3.45	63.25	7.40	3.43
15	n-C_3H_7	n-C_3H_7	64	$C_{45}H_{62}N_2O_{13}$	64.38	7.44	3.34	64.36	7.89	3.70
16	n-C_4H_9	n-C_4H_9	82	$C_{47}H_{66}N_2O_{13}$	65.10	7.67	3.23	64.87	7.84	3.51
17	i-C_4H_9	i-C_4H_9	55	$C_{47}H_{66}N_2O_{13}$	65.10	7.67	3.23	64.20	7.76	3.37
18	n-C_5H_{11}	n-C_5H_{11}	80	$C_{49}H_{70}N_2O_{13}$	65.75	7.88	3.13	66.32	7.37	2.82
19	$CH_2CH{=}CH_2$	$CH_2CH{=}CH_2$	82	$C_{45}H_{58}N_2O_{13}$	64.73	7.00	3.35	64.57	7.23	3.35
20	$CH_2C_6H_5$	$CH_2C_6H_5$	60	$C_{53}H_{62}N_2O_{13}$	68.07	6.68	3.00	66.97	7.09	3.30
21	CH_3	C_2H_5	64	$C_{42}H_{56}N_2O_{13}$	63.30	7.08	3.51	62.73	7.19	3.64
22	CH_3	n-C_3H_7	55	$C_{43}H_{58}N_2O_{13}$	63.68	7.21	3.45	63.42	7.58	3.49
23	CH_3	i-C_3H_7	68	$C_{43}H_{58}N_2O_{13}$	63.68	7.21	3.45	62.84	7.32	3.54
24	CH_3	n-C_4H_9	63	$C_{44}H_{60}N_2O_{13}$	63.59	7.28	3.37	63.06	7.77	3.44
25	CH_3	t-C_4H_9	27.5	$C_{44}H_{60}N_2O_{13}$	63.59	7.28	3.37	63.79	7.44	3.47
26	C_2H_5	n-C_3H_7	89	$C_{44}H_{60}N_2O_{13}$	63.59	7.28	3.37	63.97	7.59	3.20
27	C_2H_5	n-C_4H_9	78	$C_{45}H_{62}N_2O_{13}$	64.42	7.45	3.34	63.99	7.46	3.53
28	n-C_3H_7	$CH_2C{\equiv}CH$	50	$C_{45}H_{58}N_2O_{13}$	64.73	7.00	3.35	64.28	7.50	3.40
29	CH_3		68	$C_{45}H_{60}N_2O_{13}$	64.57	7.23	3.35	65.20	7.29	3.45
30	CH_3		70	$C_{46}H_{62}N_2O_{13}$	64.92	7.34	3.29	64.57	8.01	3.51
31	C_2H_5	C_6H_5	63	$C_{47}H_{58}N_2O_{13}$	65.72	6.81	3.26	64.80	7.01	3.55
32	CH_3	$CH_2C_6H_5$	84	$C_{47}H_{58}N_2O_{13}$	65.72	6.81	3.26	65.14	7.39	3.71
33	CH_3	CH_2CH_2OH	17	$C_{45}H_{56}N_2O_{14}$	63.66	6.65	3.30	62.61	7.13	3.34
34	C_2H_5	CH_2CH_2OH	26	$C_{43}H_{58}N_2O_{14}$	62.45	7.07	3.39	61.60	7.40	3.06
35	CH_3	CH_2CH_2CN	20	$C_{44}H_{55}N_3O_{13}$	63.37	6.65	5.04	62.62	7.40	4.60
36	C_2H_5	$CH_2CH_2N(C_2H_5)_2$	64	$C_{47}H_{67}N_3O_{13}$	63.99	7.66	4.76	63.00	7.88	4.44
37	CH_3	$(CH_2)_2N(CH_2)_2N(C_2H_5)_2$ | C_2H_5	30	$C_{50}H_{74}N_4O_{13}$	63.94	7.94	5.97	63.95	8.22	5.65
38	CH_2CH_2Cl	CH_2CH_2Cl	32.5	$C_{43}H_{56}Cl_2N_2O_{13}$	58.70	6.41	3.18 (Cl, 8.06)	58.86	6.91	3.30 (Cl, 8.15)
39		$-(CH_2)_4-$	90	$C_{43}H_{56}N_2O_{13}$	63.84	6.98	3.46	63.63	7.40	3.03
40		$-CH(CH_3)(CH_2)_2CH(CH_3)-$	38	$C_{45}H_{60}N_2O_{13}$	64.57	7.23	3.35	64.00	7.75	3.60
41		$-CH(CH_2OH)(CH_2)_2CH(CH_2OH)-$	50	$C_{45}H_{60}N_2O_{15}$	62.20	6.96	3.22	61.64	7.51	3.40
42		$-CH(COOC_2H_5)(CH_2)_2CH(COOC_2H_5)-$	40	$C_{49}H_{64}N_2O_{17}$	61.78	6.73	2.94	61.64	6.83	3.10
43		$-(CH_2)_5-$	80	$C_{44}H_{58}N_2O_{13}$	64.21	7.10	3.40	63.03	7.62	3.68
44		$-(CH_2)_2CH(CH_3)(CH_2)_2-$	52	$C_{45}H_{60}N_2O_{13}$				64.60	7.67	3.24
45		$-(CH_2)_4CH(CH_3)-$	69	$C_{45}H_{60}N_2O_{13}$	64.24	7.19	3.32	64.10	7.68	3.09
46		$-(CH_2)_6-$	82	$C_{45}H_{60}N_2O_{13}$	64.24	7.19	3.33	64.37	7.35	3.38
47		$-(CH_2)_2O(CH_2)_2-$	78	$C_{43}H_{56}N_2O_{14}$	62.60	6.84	3.40	63.22	6.22	3.37
48		$-(CH_2)_2OCH_2CH(CH_3)-$	75	$C_{44}H_{58}N_2O_{14}$	63.00	7.02	3.34	62.98	7.33	3.37
49		$-CH(CH_3)CH_2OCH_2CH(CH_3)-$	56	$C_{45}H_{60}N_2O_{14}$	63.36	7.09	3.28	61.64	7.02	2.95

[a] Melting points were indefinite. [b] No attempts were made to improve the yields.

B are generally not well defined owing to gradual decomposition during the heating.

In Table I are listed the rifamycinamides and in Table II the rifamycinhydrazides which have been prepared for biological evaluation. All these derivatives were tested for *in vitro* antibacterial activity: Tables III and IV report the minimal inhibitory concentrations against a limited number of gram-positive and gram-negative bacteria and against *Mycobacterium tuberculosis*. A number of them were tested also for the *in vivo* activity in experimental infection and for acute toxicity in mice. These results are reported in Table V.

TABLE II
RIFAMYCIN HYDRAZIDES



Compd.[a]	R_1	R_2	R_3	Yield,[b] %	Formula	Calcd., % C	Calcd., % H	Calcd., % N	Found, % C	Found, % H	Found, % N
50	H	H	H	10	$C_{39}H_{51}N_3O_{13}$	60.84	6.67	5.45	61.12	7.20	5.80
51	H	H	C_6H_5	70	$C_{45}H_{55}N_3O_{13}$	63.89	6.55	4.96	63.13	6.94	5.10
52	CH_3	CH_3	CH_3	97.5	$C_{42}H_{57}N_3O_{13}$	62.13	7.07	5.17	62.27	7.31	4.80
53	CH_3	C_2H_5	C_2H_5	62.9	$C_{44}H_{61}N_3O_{13}$	62.91	7.31	5.00	62.50	7.53	4.80
54	CH_3	n-C_3H_7	n-C_3H_7	75.6	$C_{46}H_{65}N_3O_{13}$	63.65	7.54	4.84	64.14	7.70	4.80
55	CH_3	n-C_4H_9	n-C_4H_9	44.1	$C_{48}H_{69}N_3O_{13}$	64.34	7.76	4.68	64.41	7.90	4.49
56	C_2H_5	CH_3	CH_3	92.0	$C_{43}H_{59}N_3O_{13}$	62.53	7.20	5.08	61.92	6.34	4.65
57	C_2H_5	C_2H_5	C_2H_5	60.2	$C_{45}H_{63}N_3O_{13}$	63.28	7.43	4.92	63.32	7.45	4.62
58	C_2H_5	n-C_3H_7	n-C_3H_7	75	$C_{47}H_{67}N_3O_{13}$	64.00	7.65	4.76	63.70	7.50	4.58
59	C_2H_5	n-C_4H_9	n-C_4H_9	74.4	$C_{49}H_{71}N_3O_{13}$	64.66	7.86	4.61	64.62	7.82	4.22
60	n-C_3H_7	CH_3	CH_3	90.0	$C_{44}H_{61}N_3O_{13}$	62.91	7.31	5.00	62.63	7.67	4.92
61	n-C_3H_7	C_2H_5	C_2H_5	71.4	$C_{46}H_{65}N_3O_{13}$	63.65	7.54	4.84	64.06	7.86	5.05
62	C_3H_7	C_3H_7	C_3H_7	59.6	$C_{48}H_{69}N_3O_{13}$	64.34	7.76	4.68	64.14	7.69	4.53
63	C_3H_7	n-C_4H_9	n-C_4H_9	68.5	$C_{50}H_{73}N_3O_{13}$	64.98	7.96	4.54	64.33	8.05	4.42
64	n-C_4H_9	CH_3	CH_3	96.5	$C_{45}H_{63}N_3O_{13}$	63.28	7.43	4.92	63.19	7.52	4.71
65	n-C_4H_9	C_2H_5	C_2H_5	64.2	$C_{47}H_{67}N_3O_{13}$	64.00	7.65	4.76	63.57	7.85	4.80
66	n-C_4H_9	n-C_3H_7	n-C_3H_7	47.4	$C_{49}H_{71}N_3O_{13}$	64.66	7.86	4.61	64.23	7.95	4.57
67	n-C_5H_{11}	CH_3	CH_3	61.0	$C_{46}H_{65}N_3O_{13}$	63.50	7.76	4.82	63.53	7.61	4.39
68	CH_3	$-(CH_2)_5-$		89.4	$C_{45}H_{61}N_3O_{13}$	63.43	7.21	4.93	63.28	7.59	4.64
69	C_2H_5	$-(CH_2)_5-$		60.5	$C_{46}H_{63}N_3O_{13}$	63.79	7.33	4.85	63.55	7.58	4.80
70	n-C_3H_7	$-(CH_2)_5-$		58.2	$C_{47}H_{65}N_3O_{13}$	64.14	7.44	4.77	63.79	7.70	4.57
71	n-C_4H_9	$-(CH_2)_5-$		63.8	$C_{48}H_{67}N_3O_{13}$	64.48	7.55	4.70	64.15	7.88	4.38
72	CH_3	$-(CH_2)_2O(CH_2)_2-$		78.6	$C_{44}H_{59}N_3O_{14}$	61.88	6.96	4.92	61.31	7.25	4.81
73	C_2H_5	$-(CH_2)_2O(CH_2)_2-$		75.5	$C_{46}H_{63}N_3O_{14}$	62.26	7.08	4.84	61.50	7.39	4.86
74	n-C_3H_7	$-(CH_2)_2O(CH_2)_2-$		50.0	$C_{48}H_{67}N_3O_{14}$	62.64	7.19	4.76	61.18	7.67	4.65
75	n-C_4H_9	$-(CH_2)_2O(CH_2)_2-$		72.1	$C_{50}H_{71}N_3O_{14}$	63.00	7.31	4.68	61.92	7.52	4.40

[a] Melting points were indefinite. [b] No attempts were made to improve the yields.

Experimental

Chemistry.—The condensation of rifamycin B with amines or hydrazines in tetrahydrofuran and in the presence of dicyclohexylcarbodiimide occurrs at room temperature; 2 hr. is necessary for completion of the reaction with the hydrazines and several hours in the case of amines. The preparation of rifamycinamides is therefore preferably carried out at the boiling point of the solvent in a period of time varying between 15 min. and 1 hr. according to the desired rifamycin derivative. The reaction was followed by thin layer chromatographic analysis on silica gel G plates. Using acetone as solvent, rifamycin B shows a very low mobility, whereas the amides and hydrazides have R_f values from 0.4 to 0.8; these variations are due to the nature of the substituents on the nitrogen. At the end of the reaction the solvent was evaporated to a small volume and, on cooling, the dicyclohexylurea crystallized out. The concentrated solution was then generally diluted with water, acidified with HCl, and extracted with benzene or carbon tetrachloride. After evaporation of the solvent, the crude rifamycin amides or hydrazides were recrystallyzed from appropriate solvents such as cyclohexane, benzene–hexane, acetone–water, or ethyl acetate. A few typical examples are reported.

Rifamycin B Amide (Table I, 1).—Nine grams (0.012 mole) of rifamycin B was suspended in 500 ml. of anhydrous tetrahydrofuran. Then 2.7 g. (0.013 mole) of dicyclohexylcarbodiimide was added followed by 31 ml. of tetrahydrofuran containing 1% of ammonia (corresponding to 0.018 mole of NH_3). The mixture was refluxed for 20 min., then cooled to about 10° and allowed to stand for 10–15 min. Rifamycinamide separated as lemon yellow crystals, which were filtered and washed with some tetrahydrofuran. From the filtrate, crystalline dicyclohexylurea was obtained by concentration to a small volume. The rifamycinamide showed, in thin layer chromatography on silica gel G plates (acetone as solvent), a major spot (R_f 0.5) with traces of rifamycin B (R_f 0.05) and of other impurities. One crystallization from ethyl acetate gave 4.1 g. (45%) of rifamycinamide chromatographically homogeneous. After several crystallizations from the same solvent and prolonged drying at 40° under vacuum, an analytical sample was obtained. It had no definite melting point: the crystals began to soften at 160° and melted completely at 180–185° dec., $[\alpha]^{20}D$ −1.8° (c 0.265, methanol), potentiometric titration in MeOH–H_2O (2:1) $pH_{1/2}$ 3.3. The ultraviolet spectrum in phosphate buffer pH 7.38 showed absorption maxima at 223 mμ (ϵ 41,260), 305 (20,670), and 428 (16, 130).

Rifamycin B Diethylamide (Table I, 14).—Rifamycin B (9 g., 0.012 mole) was suspended in 250 ml. of anhydrous tetrahydrofuran. Then 2.7 g. (0.013 mole) of dicyclohexylcarbodiimide was added followed by 1.4 ml. (0.014 mole) of diethylamine. The solution was refluxed for 15 min. then concentrated to one-fifth of its initial volume and allowed to stand for 3 hr. at 4°. The dicyclohexylurea was removed by filtration. The filtrate was poured into about ten times its volume of water acidified with HCl and the precipitate was extracted twice with 200 ml. of benzene. The organic phase was dried (Na_2SO_4) and, after

TABLE III
ANTIBACTERIAL ACTIVITY OF THE RIFAMYCIN AMIDES

	Minimal inhibitory concentration, γ/ml.[a]								
Compd.	*Micrococcus aureus* ATCC 6538	*Streptococcus faecalis* ATCC 10541	*Streptococcus hemolyticus* C 203	*Bacillus subtilis* ATCC 6633	*Proteus vulgaris* ATCC 881	*Escherichia coli* ATCC 10536	*Klebsiella pneumoniae* ATCC 10031	*Pseudomonas aeruginosa* ATCC 10145	*Mycobacterium tuberculosis* H37RV
1	1.5	9.2	0.18	12.5	>200	>200	200	>200	0.009
2	0.16	1	6.2	0.06	>200	200	200	200	0.15
3	0.08	0.5	0.025	3.1	>200	150	200	200	0.15
4	0.25	1.2	0.3	3.1	>200	>200	200	200	0.18
5	0.16	1	0.06	3.1	>200	>200	200	200	0.075
6	0.15	1.2	0.3	3.1	>200	>200	200	200	0.09
7	0.15	0.6	1	3.1	>200	>200	200	>200	0.18
8	0.1	0.75	0.12	3.1	>200	>200	>200	>200	0.01
9	0.25	0.35	0.12	2	>200	>200	>200	>200	0.15
10	0.3	0.25	0.2	1.5	>200	>200	>200	200	0.005
11	0.12	0.16	0.1	1.5	>200	>200	>200	>200	0.15
12	0.075	0.6	0.045	6.2	>200	>200	>200	>200	0.09
13	0.06	0.75	0.01	1.5	100	100	50	100	...
14	0.01	0.08	0.0075	0.75	25	6.2	25	50	0.075
15	0.006	0.06	0.006	0.25	12.5	6.2	25	12.5	0.18
16	0.003	0.03	0.012	0.06	12.5	6.2	25	50	0.02
17	0.0012	0.012	0.006	0.045	6.2	6.2	25	25	0.3
18	0.002	0.02	0.025	0.09	6.2	12.5	12.5	25	0.3
19	0.005	0.05	0.003	0.18	12.5	6.2	12.5	50	0.37
20	0.003	0.03	0.015	0.12	25	12.5	25	25	0.37
21	0.009	0.07	0.009	0.75	12.5	12.5	25	50	0.37
22	0.01	0.04	0.006	0.18	25	12.5	25	50	0.09
23	0.002	0.02	0.006	0.09	6.2	6.2	12.5	50	0.18
24	0.003	0.05	0.0025	0.37	12.5	6.2	25	50	0.37
25	0.0025	0.02	0.004	0.18	6.2	6.2	25	50	0.37
26	0.0075	0.05	0.005	0.37	25	6.2	25	50	0.09
27	0.0015	0.02	0.006	0.045	6.2	6.2	12.5	50	0.18
28	0.003	0.06	0.0015	0.09	25	6.2	25	50	0.37
29	0.006	0.05	0.005	0.37	25	6.2	25	25	0.09
30	0.006	0.025	0.009	0.18	12.5	6.2	25	25	0.09
31	0.003	0.04	0.006	0.37	6.2	6.2	25	25	0.18
32	0.005	0.07	0.006	0.37	12.5	12.5	12.5	50	0.37
33	0.1	1.2	0.06	5	>100	>100	>100	>100	0.15
34	0.025	0.55	0.09	1.55	>100	100	100	>100	0.09
35	0.09	0.75	0.09	1.5	>100	100	100	>100	0.09
36	0.12	0.5	0.03	3.1	200	25	50	50	0.075
37	0.03	0.75	0.09	0.75	>100	25	100	>100	0.18
38	0.05	1.5	0.03	0.37	>100	100	>100	>100	0.09
39	0.045	0.37	0.005	1.5	50	25	25	50	0.01
40	0.005	0.03	0.005	0.18	6.2	6.2	12.5	100	0.37
41	0.07	1.3	0.025	3.1	>100	>100	>100	>100	0.18
42	0.005	0.2	0.012	0.75	100	50	50	>100	0.37
43	0.0075	0.1	0.005	0.75	25	6.2	25	25	0.005
44	0.005	0.045	0.01	0.37	12.5	6.2	25	50	0.37
45	0.005	0.03	0.01	0.18	25	6.2	25	50	0.18
46	0.004	0.03	0.01	0.3	6.2	6.2	12.5	25	0.09
47	0.3	1.2	0.045	1.5	200	50	50	200	0.1
48	0.01	0.5	0.0037	0.18	>100	25	50	100	0.09
49	0.01	0.09	0.015	0.6	50	12.5	50	100	0.09
Rifamycin SV[b]	0.005	0.09	0.0025	0.075	25	12.5	25	25	0.05

[a] Minimum inhibitory concentration is the lowest concentration of antibiotic that prevents visible growth after 18-hr. incubation.
[b] For comparison.

addition of 150 ml. of hexane, was concentrated to about 400 ml. A slight precipitate, which was chromatographically impure, was removed by filtration and the filtrate was concentrated to a small volume. The yellow-orange precipitate so obtained weighed 9.0 g. (93% yield) and was homogeneous in thin layer chromatography (R_f 0.6 in the system mentioned before). A sample crystallized twice from benzene–hexane and dried for 2 days at 40° under vacuum gave analytical results in correspondence with the calculated values. It had no definite melting point but began to soften at 140° and melted completely at 170° dec.; $[\alpha]^{20}D$ −48.7° (*c* 0.4, methanol); potentiometric titration in MeOH–H_2O (2:1) pH $_{1/2}$ 3.7; ultraviolet spectrum in phosphate buffer (pH 7.38): 222 mμ (ε 42,820), 302 (20,770), and 421 (16,200)

Rifamycin B Dimethylamylhydrazide (Table II, 67). —Rifamycin **B** (9 g., 0.012 mole) was suspended in 500 ml. of tetrahydrofuran. Then 2.7 g. of dicyclohexylcarbodiimide (0.013 mole) was added followed by 1.70 g. (0.013 mole) of N,N-dimethyl-N'-amylhydrazine dissolved in 500 ml. of tetrahydrofuran. After the addition, carried out in 30 min. with vigorous stirring at room temperature, the mixture was allowed to stand for 2 hr., then concentrated to 80 ml. The precipitated dicyclohexylurea was filtered off, the filtrate was poured into 1000 ml. of water

TABLE IV
ANTIBACTERIAL ACTIVITY OF THE RIFAMYCIN HYDRAZIDES

	Minimal inhibitory concentration, γ/ml.[a]								
Compd.	*Micrococcus aureus* ATCC 6538	*Streptococcus faecalis* ATCC 10541	*Streptococcus hemolyticus* C 203	*Bacillus subtilis* ATCC 6633	*Proteus vulgaris* ATCC 881	*Escherichia coli* ATCC 10536	*Klebsiella pneumoniae* ATCC 10031	*Pseudomonas aeruginosa* ATCC 10145	*Mycobacterium tuberculosis* H37RV
50	0.08	0.7	0.06	3.1	>200	200	>200	200	0.15
51	0.1	1	0.05	3.1	>200	200	>200	200	0.037
52	0.006	0.05	0.002	0.18	12.5	6.2	25	100	0.18
53	0.0015	0.02	0.005	0.09	12.5	12.5	12.5	100	0.37
54	0.001	0.015	0.0015	0.09	20	6.2	5	50	0.37
55	0.0012	0.01	0.002	0.045	12.5	6.2	0.37	25	0.09
56	0.002	0.03	0.0015	0.18	6.2	6.2	25	50	0.18
57	0.001	0.02	0.002	0.02	12.5	6.2	3.1	50	0.15
58	0.0012	0.015	0.0012	0.045	25	6.2	0.75	50	0.18
59	0.0012	0.01	0.0005	0.045	12.5	12.5	0.37	25	0.09
60	0.003	0.02	0.0025	0.18	5	3.1	25	50	0.15
61	0.001	0.02	0.0015	0.02	25	6.2	25	50	0.15
62	0.003	0.02	0.003	0.06	25	6.2	25	25	0.09
63	0.003	0.02	0.0025	0.03	25	12.5	12.5	25	0.09
64	0.001	0.018	0.0045	0.045	6.2	1.5	12.5	50	0.15
65	0.0018	0.01	0.01	0.02	12.5	6.2	25	50	0.18
66	0.002	0.02	0.0025	0.045	25	6.2	25	25	0.09
67	0.0015	0.01	0.007	0.045	6.2	2.5	12.5	50	0.18
68	0.0025	0.02	0.01	0.09	12.5	6.2	25	25	0.09
69	0.0025	0.02	0.01	0.09	6.2	6.2	25	25	0.045
70	0.002	0.01	0.01	0.045	12.5	6.2	25	25	0.045
71	0.002	0.01	0.007	0.045	6.2	6.2	25	25	0.09
72	0.005	0.18	0.015	0.37	100	25	50	>100	0.18
73	0.02	0.18	0.0012	0.75	50	6.2	50	100	0.37
74	0.007	0.06	0.005	0.37	25	6.2	50	100	0.37
75	0.02	0.18	0.02	1.5	>100	50	>100	>100	1.5
Rifamycin SV[b]	0.005	0.09	0.0025	0.075	25	12.5	25	25	0.05

[a] See footnote *a* in Table III. [b] For comparison.

acidified with HCl and extracted with ethyl acetate. The extract was dried (Na_2SO_4), filtered, concentrated to 40–60 ml. and poured into 1000 ml. of petroleum ether (b.p. 40–60°). The crude hydrazide, precipitated in the form of a yellow-orange amorphous powder, was collected and dissolved in 1600 ml. of benzene, then 4800 ml. of cyclohexane was added and the solution was concentrated to about 200 ml. The first crop, chromatographically impure, was discarded by filtration and the filtrate was concentrated to about 200 ml. The precipitated dimethylamylhydrazide of rifamycin B was collected, washed with cyclohexane, and dried for 2 days at 40°. It weighed 8.0 g. (61% yield) and was homogeneous in thin layer chromatography (R_f 0.65, acetone as solvent). A sample crystallized twice from ethyl acetate–hexane and dried for 2 days at 40° gave analytical results in agreement with the calculated values. It softened at 130° and melted completely at 150–155° dec.; $[\alpha]^{20}D$ −72.4° (*c* 0.166, methanol); potentiometric titration in MeOH–H_2O (3:1) pH $\frac{1}{2}$ 4.5; ultraviolet spectrum in phosphate buffer (pH 7.38): 222 mμ (ε 41,820), 302 (21,180), and 421 (16,180).

Biological Tests. *In Vitro* **Activity.**—The antimicrobial activity of these new rifamycins was assayed by determining the minimum inhibitory concentrations against gram-positive and gram-negative bacteria using the serial dilution technique in nutrient broth. The bacterial inoculum for each strain was standardized by turbidimetric assay in order to have comparative values for the different derivatives. The minimal inhibitory concentration was the lowest concentration of antibiotic which prevented visible growth after 18-hr. incubation at 37°. A comparative study of these derivatives was extended also to their antituberculous activity. The inoculum consisted of 0.5–1% of a 7–9 day culture in Dubos medium of *Mycobacterium tuberculosis* $H_{37}Rv$; the tests were carried out in Kirschner medium and observations were made after 7-days incubation at 37°.

In Vivo **Activity.**—The therapeutic activity of a certain number of rifamycin amides and rifamycin hydrazides was tested in acute experimental staphylococcal infections in mice. The sodium salt of rifamycin amides or rifamycin hydrazides was administered by subcutaneous and oral routes in 2 daily doses to groups of 10 animals per dose. After 7-days treatment, deaths were recorded and a median therapeutic dose (ED_{50}) was determined.

Acute Toxicity.—The acute toxicity of the derivatives to be tested for the *in vivo* activity was determined in mice. The amides and hydrazides of rifamycin B, as sodium salts, were administered by intravenous route to groups of 10 animals. All animals were observed for 96 hr. and deaths were recorded. The Litchfield and Wilcoxon method was used to calculate the LD_{50} values.

Discussion

The purpose of the present work was to compare the antibacterial activities of derivatives of rifamycin B, in which the carboxyl group has been blocked by conversion into amides or hydrazides, with the activity of rifamycin SV, which is the final product of "activation" of rifamycin B after the elimination of glycolic acid. The data reported in Tables IV and V confirm our hypothesis that substitution in the inactive rifamycin B of the free carboxyl group results in the appearance of high activity, in several cases of the same order as that of rifamycin SV.

In the series of the amides, the unsubstituted rifamycin amide (**1**) has only a slight activity against gram-positive and no activity against gram-negative bacteria. The N-monosubstituted amides (**2–12**) show a certain activity against gram-positive and no activity against gram-negative bacteria. The other rifamycin amides are disubstituted and the greater part of them possess an exceptionally high antibacterial activity against gram-positive bacteria and a limited, although not negligible, activity against gram-negative bacteria. Among the disubstituted amides, the less active compounds are those containing hydroxyl (**33**, **34**, **41**),

TABLE V
In Vivo ACTIVITY AND ACUTE TOXICITY OF SOME AMIDES AND HYDRAZIDES OF RIFAMYCIN B

Compd.	ED_{50}, mg./kg. (staphylococcal infections in mice) Oral	ED_{50}, mg./kg. (staphylococcal infections in mice) S.c.	LD_{50}, mg./kg. i.v. (in mice)
3	>110	>21.0	719 (685.4–755.7)
8	>80	>25.0	350 (381.5–321.4)
13	>110	~15.5	564 (513.3–621.1)
14	21 (18.6–23.7)	2.6 (2.28–2.96)	429 (360.5–510.5)
15	25 (18.9–33)	4.5 (3.1–6.25)	175 (142.2–215.2)
16	34 (23.4–49.3)	10.5 (6.78–16.27)	141 (129.3–153.6)
17	14 (11–17.8)	4.5 (3.3–6.3)	152 (143.3–161.1)
19	45 (36–56.2)	8.6 (6.9–10.5)	245 (214.9–279.3)
20	~55	~20.0	185 (141.2–242.3)
21	>80	~16.0	720 (666.6–777.6)
22	70 (62.5–78.4)	4.5 (3.1–6.5)	368 (306.6–441.6)
23	50 (42–59.5)	13.0 (8.53–19.76)	275 (308.0–245.5)
24	34 (27.4–42.1)	4.4 (3.3–5.9)	265 (250.0–280.9)
26	34 (28.1–41.1)	4.6 (3.3–6.3)	230 (184.0–287.5)
27	29 (18–46.4)	7.4 (5.06–10.8)	205 (178.2–235.7)
29	43 (34.9–52.8)	5.5 (4.2–7.3)	283 (268.2–298.5)
30	35 (30.1–40.6)	6.0 (4.47–8.04)	212 (195.3–230.0)
31	36 (30.2–42.8)	4.4 (3.2–5.9)	257 (244.2–270.3)
32	48 (41.3–55.68)	6.4 (4.78–8.57)	222 (211.4–233.1)
39	~70	~9.2	680 (741.2–623.8)
40	17.4 (15.1–20.1)	5.35 (4.55–6.31)	233 (195–277)
43	~12.3	~4.0	285 (263.8–307.8)
46	23 (20.7–25.5)	4.35 (3.77–5.03)	220 (207–233)
47	>110	>20.0	925 (868.5–985.1)
48	>80	>16.0	~950
49	>80	~15.0	695 (655–736)
52	58 (42.9–72.3)	7.4 (5.48–9.9)	640 (581.8–704.0)
53	35.5 (28.5–39.1)	2.8 (1.77–4.42)	350 (312.5–392.0)
54	28.3 (25.7–31.2)	~3.5	250 (223.2–280.0)
55	~16	~3.7	198 (159.6–245.5)
56	47 (30.9–71.4)	2.7 (1.68–4.32)	470 (449.7–491.1)
57	22 (15.4–31.2)	2.0 (1.39–2.86)	302 (284.9–320.1)
58	20 (17.8–22.5)	4.0 (3.5–4.57)	210 (185.8–237.3)
59	24.6 (21.7–27.95)	5.67 (4.96–6.45)	188 (163.4–216.2)
60	25 (18.8–33.25)	2.9 (1.93–4.35)	272 (261.5–282.8)
61	19.5 (13.4–28.2)	1.7 (1.04–2.75)	192 (165.5–222.7)
62	24.6 (21.7–28)	6.5 (5.89–7.17)	240 (214.2–268.8)
63	20 (16.8–23.7)	6.5 (5.92–7.13)	172 (143.3–206.4)
64	19 (13.2–27.36)	2.3 (1.51–3.49)	212 (196.2–228.9)
65	>75	12.5 (5.7–27.5)	129 (118.8–139.9)
66	~24.6	~6.5	209 (184.9–236.1)
67	16.5 (11.5–23.6)	2.5 (1.92–3.25)	146 (136.4–156.2)
68	~70	3.48 (3.02–4.02)	280 (256.8–305.2)
69	37.3 (32.9–42.4)	3.0 (2.63–3.49)	190 (169.6–212.8)
70	26.4 (22.4–31)	~7.0	185 (174.5–196.1)
71	34.8 (31–39.1)	5.0 (4.37–5.72)	149 (138.4–172.8)
72	>80	>15.0	865 (823.8–908.2)
Rifamycin SV[a]	74 (60–90)	17.7 (11–26)	550 (482–627)

[a] For comparison.

cyano (**35**), chloro (**38**), diethylamino (**36**, **37**) or carbethoxy (**42**) groups at the alkyl chain. In the group of heterocyclic amides, the cyclic oxygen of the rifamycin morpholides (**47–49**) also seems to have a negative influence on the antibacterial activity.

Similar correlations have been found in the series of hydrazides. The unsubstituted rifamycin hydrazide (**50**) and the N-monosubstituted hydrazide (**51**) show a certain activity only against gram-positive but no activity against gram-negative bacteria. All the other N,N,N′-trisubstituted hydrazides show exceptionally high activity against gram-positive and a certain activity against gram-negative bacteria. In this series the less active compounds appear to be the N,N-diethylenoxy-N′-alkylhydrazides (**72–75**) bearing a cyclic oxygen in the molecule; this behavior is very similar to that mentioned above for the rifamycin morpholides. All the derivatives inhibit the growth of *M. tuberculosis* at very low concentrations, but the correlations between the activity and nature of the substituents do not follow the same pattern as in the case of the activity against the other microorganisms tested.

In conclusion, the *in vitro* activity of the N,N-disubstituted amides and of the N,N,N′-trisubstituted hydrazides, with no other functional groups on the substituents, is of the same order as that of rifamycin SV and in some cases a little higher.

The *in vivo* activity of this series of new derivatives of rifamycin B in experimental infections in mice was

tested by administering the substances orally and subcutaneously. It is known that rifamycin SV is poorly absorbed by the oral route and that the rapid elimination through the bile prevents the appearance of therapeutic blood levels when given orally.[11] For these reasons this antibiotic is used in therapy only by parenteral administration. Therefore in testing new derivatives of rifamycin B, absorption from intestine and concentration in bile were systematically investigated for each active compound. Laboratory studies on these two aspects of the pharmacology of the new rifamycins are in progress and will be reported later. A preliminary screening of the oral and subcutaneous activity of these derivatives against experimental infections was thought to give some indirect information on this aspect of the problem.

The results given in Table V lead to the following considerations. The monosubstituted amides (**3**, **8**) give very poor *in vivo* protection in agreement with the slight *in vitro* activity. With the exception of the rifamycin morpholides, the other disubstituted rifamycinamides, which show exceptionally high *in vitro* activity, have a good protective effect when administered parenterally. By the oral route the ED_{50} values are always many times higher than the s.c. ED_{50}. It is interesting to note that in the homologous series the ratio ED_{50} oral/ED_{50} s.c. is generally higher for the compounds bearing the shorter alkyl chains. For example, this ratio is >8.0 for the dimethyl-, 8.0 for the diethyl-, 5.55 for the dipropyl-, and 3.2 for the di-*n*-butylrifamycinamide. Since in a homologous series the water solubility decreases and the lipid solubility increases with the increase of the carbon chains on the nitrogen, the ratio of lipid solubility–water solubility of the different rifamycinamides can be considered of importance for gastrointestinal absorption.

As far as the *in vivo* activity of the rifamycin hydrazides is concerned, the situation is parallel to that of the rifamycinamides. With the exception of the N,N-diethylenoxy-N′-methylhydrazide (**72**), which is slightly active, all the other trisubstituted hydrazides are effective in protecting the infected animal at low doses when administered subcutaneously. By the oral route effectiveness appears at higher doses.

The acute toxicity of these derivatives can be judged from the data in Table V. In a series of homologs the toxicity increases with the molecular weight; for example, dimethylamide, 654; diethylamide, 429; dipropylamide, 175; and dibutylamide, 141. Other examples of this correlation can be observed in the series of rifamycin hydrazides. The less toxic derivatives are those containing a morpholino group, both in the series of amides (**47**–**49**) and in the series of hydrazides (**72**). As mentioned before these derivatives show a limited therapeutic efficacy in experimental infections.

In conclusion, comparison of the *in vitro* and *in vivo* activities and toxicity of the amides and hydrazides of rifamycin B with the corresponding data of rifamycin SV (which are included in Table III–V) indicates that a great number of the new derivatives here reported show favorable properties as potential therapeutic agents. Obviously, the comparative evaluation of these derivatives will require further pharmacological and toxicological studies. Laboratory investigations on some members of the series are in progress.

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D-Aspartyl[1]-valyl[5]-phenylalanine[8] Amide Angiotensin II

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D-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine amide, an analog of bovine angiotensin II, was synthesized, and results of enzymatic studies with leucine aminopeptidase, trypsin, chymotrypsin, and carboxypeptidase support its structure. The peptide increased rat blood pressure with an average potency of 1/300th that of asparaginyl[1]-valyl[5] angiotensin II; a threefold increase in the duration of action was exhibited at dose levels which gave an equivalent absolute response.

Extensive studies of structure–activity relationships in the angiotensin series have been reviewed by Schwyzer[1] as well as by Page and Bumpus.[2] Alteration of the C-terminus of angiotensin II resulted in a striking quantitative change in biological activity, whereas modification of the N-terminus had little influence. Conversion of the C-terminal carboxyl group to a carboxamide function decreased biological activity to 1/30, and either elimination of C-terminal L-phenylalanine or its replacement by D-phenylalanine abolished activity. Substitution of the N-terminal L-aspartic acid residue by L-asparagine had no deleterious effect on activity and elimination of L-asparagine decreased activity to only 1/2. Replacement of the N-terminal amino acid by D-asparagine actually increased activity. The C-terminus of angiotensin II, therefore, appears to be of greater importance for biological activity than the N-terminus.

Recently, efforts have been directed toward the elucidation of the mode of physiological inactivation of angiotensin II. Information from experiments *in vitro* suggested that specific aminopeptidases are pri-

(1) R. Schwyzer, *Pure Appl. Chem.*, **6**, 265 (1963).
(2) I. H. Page and F. M. Bumpus, *Physiol. Rev.*, **41**, 331 (1961).