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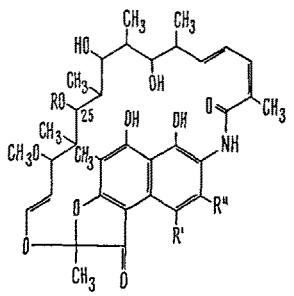
SPECIALIA

Desacetyl-Rifamycins: Preparation and Antibacterial Properties[1]

In our attempts to assay the reactivity of N-substituted 3-aminomethyl rifamycins[2] against alkaline cyanides in lower alkanols we have isolated new rifamycins which, on a careful examination, were revealed to be the corresponding 25-desacetyl derivatives (I).



I R=H
II R=COCH$_3$

It was soon realized that the basic medium induced by the cyanide was responsible for the transformation. Thus the same reaction could be reproduced by treatment of a number of rifamycins with bases.

We wish to report here a suitable preparation method along with physico-chemical properties and antimicrobial activity data of some desacetyl-rifamycins. As a general method (procedure A) these derivatives were obtained by treating the selected rifamycin (II) in 20 volumes of 5% NaOH solution in aqueous ethanol (50:50) at room temperature. The reaction was generally complete within half an hour. The solution was worked up after dilution with 2 volumes of iced water, by extracting the desacetyl-derivative with chloroform or ethyl acetate, adjusting the

[1] Rifamycins LV [Rifamycins LIV: S. Föresz, R. Scotti, R. Pallanza and E. Mapelli, Arzneimittel-Forsch. *17*, 726 (1967)].
[2] N. Maggi, V. Arioli and P. Sensi, J. med. Chem. *8*, 790 (1965).

Table I.

Compound	R′ (see formula)	R″	Method of preparation	Yields %	Electronic absorption data[a] λ_{max} (nm)	ε	TLC[b] Rf$_{rel}$[c]
Ia	OH	CH_2–N(C_2H_5)$_2$	A	74	315 448	17,300 13,700	0.63
Ib	OH	CH_2–N⟨ ⟩	A	68	315 448	17,100 13,000	0.64
Ic	OH	CHO	A	72	325 490	14,400 13,200	0.70
Id	OH	CH=N–C_6H_5	B (from Ic)	54	328 490	14,300 12,100	0.89
Ie	OH	CH=N–N(C_3H_7)$_2$	A	48	342 478	25,300 15,500	0.83
If	OH	CH=N–N⟨ O ⟩	A	75	335 475	24,900 14,400	0.80
Ig	OH	CH=N–N⟨ N–CH_3 ⟩	A	77	334 475	27,000 15,400	0.56
Ih	OH	CH=N–OCH_3	A	60	328 470	22,200 14,900	0.87
Ii	N=N–C(=NH)–NH_2	H	A	69	284 358 528	22,800 16,600 17,300	0.85
Il	OCH_2CON(C_2H_5)$_2$	H	A	71	303 425	20,800 14,200	0.79

[a] Registered in buffered solution (pH = 7.38). Maxima under 280 nm have not been reported. [b] Thin-layer chromatography on silica-gel G buffered at pH = 6.0 (McIlvaine buffer); running distance: 10 cm. The solvent was a chloroform-methanol mixture 9:1; only in 2 cases (Id and Il) it was a 1:1 mixture of acetone-$CHCl_3$. [c] Ratio between Rf value of the product to that of the parent rifamycin.

pH according to the ionization properties of the rifamycin considered. After evaporation of the organic layer in vacuo the crude product was crystallized from a suitable solvent. When the rifamycin, for instance a Schiff base of 3-formyl rifamycin SV[3], was found to be sensitive to the basic conditions, the derivative was obtained by an indirect method (procedure B), starting from the suitable pre-desacetylated, more stable rifamycin.

Table I reports the preparative procedure followed and some physico-chemical data for a number of desacetyl-rifamycins which were prepared for antibacterial testing purposes. As a criterion of identification TLC data are given, in comparison with those of the corresponding acetylated compounds (Rf_{rel}).

Evidence that the reaction resulted in splitting of the acetyl residue at C-25 was furnished by NMR-spectra, whose features are exemplified by comparing the spectrum of Ig with that of the corresponding rifamycin IIg (rifampicin[4]). In fact, in the former, the signal of the acetoxylic methyl at C-25 ($\tau = 7.93$) is lacking; the C-25-H signal shifts from 5.03 τ to 6.5–6.0 τ, in agreement with the expected displacement[7]. Moreover, other minor spectral variations are observable, which are connected with the probable conformational change induced by desacetylation; namely the 34-methyl signal displaced from 10.32[8] to 9.73 τ and the methoxy signal from 6.95 to 6.82 τ.

IR-spectra of the reported rifamycins show, as the main difference, the disappearance of the carbonyl band (about 1710 cm^{-1}) attributed to the ester function[8].

That the only modification is due to the lack of the acetyl group is confirmed by the unchanged electronic absorption spectra and polarographic behaviour and by elemental analysis data. Desacetyl-rifamycins are well crystallizable compounds. Their colour does not differ substantially from that of the parent rifamycins, whereas the water solubility is, as a rule, higher.

The in vitro antibacterial activity of desacetyl-rifamycins is reported in Table II, in which comparative data for the corresponding acetylated rifamycins are also given. For all the compounds synthesized, a marked increase of M.I.C. against gram-positive microorganisms is evident although the activity in se is still high. On the other hand, the activity against gram-negative ones with the exception of the 2 Mannich derivatives (Ia, b), remains only slightly affected. A different picture arises on examination of the M.I.C. data against *M. tuberculosis* for which there seems to be no rule. In most compounds the activity is reduced from $^1/_2$–$^1/_{20}$ of that of the original compounds, with the

[3] N. MAGGI, G. G. GALLO and P. SENSI, Farmaco (Ed. Sci.) *22*, 316 (1967).

[4] Rifampicin is the common name adopted for 3-(4-methyl-1-piperazinyl-iminomethyl)-rifamycin SV[5,6], an orally active antibiotic discovered in Lepetit Research Laboratories, which is presently in clinical experimentation.

[5] N. MAGGI, R. PALLANZA and P. SENSI, Antimicrob. Ag. Chemother. *1965*, 765.

[6] N. MAGGI, C. R. PASQUALUCCI, R. BALLOTTA and P. SENSI, Chemotherapia *11*, 285 (1966).

[7] J. N. SHOOLERY and M. T. ROGERS, J. Am. chem. Soc. *80*, 5121 (1958).

[8] Unpublished data.

Table II.

Compound (see Table I)	Minimal inhibitory concentration (μg/ml)[a]							
	S. aureus ATCC 6538	*Str. hemol.* C 203	*Str. faecalis* ATCC 10541	*Proteus v.* ATCC 881	*E. coli* ATCC 10536	*K. pneumoniae* ATCC 10031	*Pseudom. aerug.* ATCC 10145	*Mycobact. tuberc.* $H_{37}Rv$ ATCC 9360
Ia	2	5	20	> 100	> 100	100	> 100	2
IIa[2]	0.02	0.05	0.4	> 100	50	25	100	0.2
Ib	0.5	5	10	> 100	> 100	20	> 100	0.1
IIb[3]	0.05	0.15	0.5	100	2	5	20	0.05
Ic	0.2	5	0.5	5	10	5	10	2
IIc[3,5]	0.002	0.02	0.02	2	2	20	10	0.1
Id	5	5	0.5	20	10	5	10	1
IId[5]	0.005	0.1	0.02	5	10	20	20	5
Ie	0.01	2	0.5	10	10	20	> 100	0.05
IIe[5]	0.002	0.2	0.05	2	5	10	20	0.05
If	0.02	0.5	0.2	10	20	20	50	0.2
IIf[5]	0.002	0.05	0.01	2	5	20	20	0.2
Ig	0.05	0.5	0.5	20	1	2	10	0.05
IIg[5,6]	0.002	0.02	0.01	5	1	5	10	0.5
Ih	0.02	0.2	0.2	2	10	5	20	0.2
IIh[5]	0.0005	0.01	0.005	2	5	10	10	0.05
Ii	0.05	0.02	0.1	2	1	2	1	0.01
IIi[9]	0.002	0.002	0.005	0.2	0.5	1	1	0.001
Il	0.2	0.05	0.5	20	10	20	50	0.5
III[10,11]	0.01	0.01	0.1	25	6	25	50	0.1

[a] M.I.C. were determined in broth by serial dilution method.

exception of compounds Id and Ig (desacetyl-rifampicin) which show higher activity and Ie and If which maintain their activity. All the compounds are currently being evaluated for the in vivo activity and toxicity. The results will be published elsewhere.

In conclusion, it must be pointed out that the new class of rifamycins described above proves the possibility of modifying the antibacterial spectrum of rifamycins by a limited chemical manipulation of the aliphatic bridge of the molecule[12]. This finding constitutes an additional evidence of the versatility of the molecule, giving a further contribution to better knowledge of the structure-activity relationship for rifamycins[13].

Riassunto. Vengono descritte la sintesi, le proprietà chimico-fisiche e l'attività antibatterica in vitro di alcune desacetil-rifamicine. Vengono discusse le variazioni osservate nello spettro antibatterico in confronto con gli analoghi acetilati al C-25.

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Laboratori Ricerche Lepetit, Milano (Italy), 15 November 1967.

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[12] H. F. Bickel, W. Knüsel, W. Kump and L. Neipp, Antimicrob. Ag. Chemother. *1966*, 352.
[13] For a review on the influence of chemical modifications upon antibacterial activity of rifamycins see: P. Sensi, N. Maggi, S. Füresz, G. Maffii, Antimicrob. Ag. Chemother. *1966*, p. 699.

Molecular Properties of Pepsin as Studied by Gel Filtration

According to Golub and Picket[1] pepsin presents, by varying the pH, molecular disorganization. By using ultracentrifugation, Dieu was able to show[2] that below pH 5.5 and between pH 7.0 and 10.0, pepsin is formed by a homogeneous molecule, while between pH 6.25 and 7.0, it appears in more than one fraction. This finding would be explained as a consequence of the existence of the native and the denatured forms of the enzyme. The same author observed that denatured pepsin undergoes an enlargement of the molecule with respect to the active form. Edelhoch[3,4] has also showed that the molecule of alkali denatured pepsin presents a dilatation probably due to a deformation of the helix of the natural molecule.

By following denaturation with alkali, temperature and iodination, Ishii and Yasuda[5] found that the value of osmotic pressure increases with respect to the active enzyme. Conformational changes of pepsin molecule above pH 5.0 were also observed by Blumenfeld et al.[6].

Recently, phenomena of polymerization and conformational changes of several enzymes have been studied by gel filtration[7–9]. It therefore seemed of interest to investigate the inactivation process of pepsin and the accompanying changes of the molecule structure, by using gel filtration.

To this end, a series of experiments were performed, in which equal amounts of pepsin were fractionated at various pHs, ranging from 6.5–7.25. Variations of the elution patterns of the protein profiles and of the enzymatic activity were taken into consideration.

For all the experiments × 2 crystallized pepsin from 'Sigma' Chemical Co. was used; gel filtration was performed on G-100 and G-75 Sephadex column (1.8 × 90 cm) in a cold room. *Tris*-phosphate buffers (0.005 *M Tris* with added H_3PO_4 to desired pH value) containing 0.15 *M* NaCl, were used. The pepsin samples were prepared by dissolving 20 mg of enzyme in 4 ml of the same buffer used to equilibrate and elute the Sephadex column. The pH measurements were done with a 'Radiometer' type TT-1. Proteins were assayed by the Lowry[10] method using bovine albumin 'Sigma' Chemical Co. as standard.

The proteolytic activity of pepsin was assayed in each fraction by using hemoglobin as substrate, in a 0.2 *M* HCl-KCl buffer pH 1.8. The hydrolysis products, separated with 5% TCA and centrifugation, were read at 280 nm.

Typical patterns of gel filtration on Sephadex G-100 at various pHs, are reported in Figure 1. It appears that pepsin, at pHs below 6.75, is localized in one symmetrical peak and still retain high enzymatic activity. At pH 6.75, the protein peak begins to appear deformed in its ascendent part which shows a net shoulder. The elution volume is decreased and consequently an increase of the molecular weight is noted. The enzymatic activity appears now strongly reduced and it is localized only in the fractions corresponding to the original, lower molecular form of the enzyme. At pH 6.85, the elution profile shows 2 distinct protein peaks; the first one is completely devoid of enzymatic activity, while the second peak still maintains a residual activity.

Increasing the pH from 6.85 to 7.25, the conversion process of the active form into the larger, inactive, molecular form is practically completed (Figure 1 D, E, F). The molecular weight of this new form of pepsin, appearing at pH 7.25, was estimated by comparing its elution volume with that of crystallized bovine albumin.

As shown in Figure 2, both alkali denatured pepsin and albumin had approximately the same elution volume and therefore appeared to have similar molecular size.

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