Waals interactions. Each of the positively charged amino groups of putrescine is surrounded by carboxyl groups from both the main and side chain of aspartic acid molecules. Within a radius of 5 Å from the amino group, a total of 10 anionic groups and 3 cationic groups are found. Similarly, the side chain carboxyl groups of aspartate are surrounded by 6 amino groups within a radius of 5 Å. This pattern of charge clustering is due to the electrostatic component of complex formation. The nonpolar atoms of the aspartic acid side chain and putrescine backbone form a layer of the structure separated by polar layers formed by carboxylates and amino groups. Putrescine amino groups form all possible hydrogen bonds in both of its complexes with aspartic and glutamic acids, whereas the carboxylates form only part of the possible hydrogen bonds. This is apparently due to the inherent flexibility in the backbone of polyamines which allows them to adopt structures suitable for optimizing stabilizing interactions with other biomolecules. These hydrogen bonds, therefore, must form part of any structural model describing polyamine interactions.

1. Savithri, H. S., Munshi, S. K., Suryanarayana, S., Divakar, S. and Murthy, M. R. N., *J. Gen. Virol.*, 1987, **68**, 1533.
2. McMahon, M. E. and Erdmann, V. A., *Biochemistry*, 1982, **21**, 5280.
3. Tabor, C. W. and Tabor, H., *Annu. Rev. Biochem.*, 1976, **45**, 285.
4. Tabor, C. W. and Tabor, H., *Annu. Rev. Biochem.*, 1984, **53**, 749.
5. Cabanne, F., Martin-Tanguy, J. and Martin, C., *Physiol. Veg.*, 1977, **15**, 429.
6. Kurland, C. G., *Cell*, 1982, **28**, 201.
7. Ramaswamy, S., Nethaji, M. and Murthy, M. R. N., *Curr. Sci.*, 1989, **50**, 1160.
8. Main, P., MULTAN 80 *A System of Computer Programs for the Automatic Solution of Crystal Structures from X-ray Diffraction Data*, 1980, Univ. of York, England and Lourain, Belgium.
9. Chandrasekar, K. and Vasantha Pattabhi, *Acta. Crystallogr.*, 1980, **B36**, 2486.
10. Takusagawa, F. and Koeityle, T. F., *Acta Crystallogr.*, 1978, **B34**, 1910.
11. Derissen, J. L., Endeman, H. J. and Peerdeman, A. F., *Acta Crystallogr.*, 1968, **B24**, 1349.
12. Bhat, T. N. and Vijayan, M., *Acta Crystallogr.*, 1978, **B34**, 2556.

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A simple method for derivatization of a complex organic compound for biological purposes

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Derivatization of an inherently complex organic compound for biological purposes often faces serious problems from the viewpoint of physical and chemical nature of the compound. The present study takes a complex molecule rifamycin B, which is in itself complicated by the presence of several functional groups, its chemical lability and extremely low solubility. The C_{21}/C_{23} functionalization has been achieved leaving aside the aromatic region. Of the two varieties of derivatives prepared, the citrate has been found to be soluble in aqueous medium, and hence more suitable for biological purposes. Immunization is also reported. This simple methodology of preparation of the water soluble citrate derivative should find general application in biochemistry provided the organic compound under consideration contains an NH_2 or OH group.

For various purposes like affinity design, antibody production, drug delivery, etc. it is often necessary to derivatize an organic compound under mild condition at a suitable position[1-7]. Keeping in view the complexities of many biologically active compounds it is often necessary to deal with every compound separately by complicated synthetic routes[1,3,8-10]. In addition to the chemical complexities, many compounds are insoluble in water adding further problems in chemical conversions as well as difficulties in biochemical experiments which are mostly carried out in aqueous medium. The physical problem of insolubility in water makes it extremely difficult to carry out further conjugation reaction of the derivative with macromolecules like proteins. Even in case the reaction can be carried out, the product becomes insoluble and hence unusable for biological experiments.

It is thus desirable to have a way out to tackle this multidimensional problem of physicochemical nature. Ideally, we should have a methodology that is i) simple so that it can be easily carried out in a biochemical laboratory, and ii) of general applicability which can be widely used for large number of compounds. In the present study we have chosen a sparingly soluble, chemically labile and biologically important compound rifamycin B (Ciba–Geigy, USA) which is also complicated in itself by the presence of several functional groups[11]. The compound has successfully been converted to functional derivatives at desired position, and it has been found that the derivative which is soluble in water has more potential for biological application like

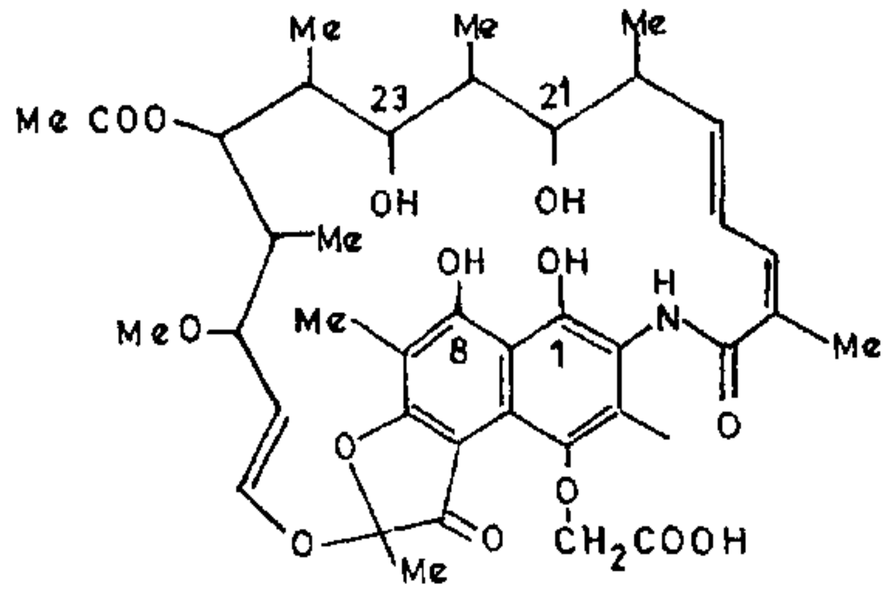
antibody raising, in comparison with the insoluble derivative.

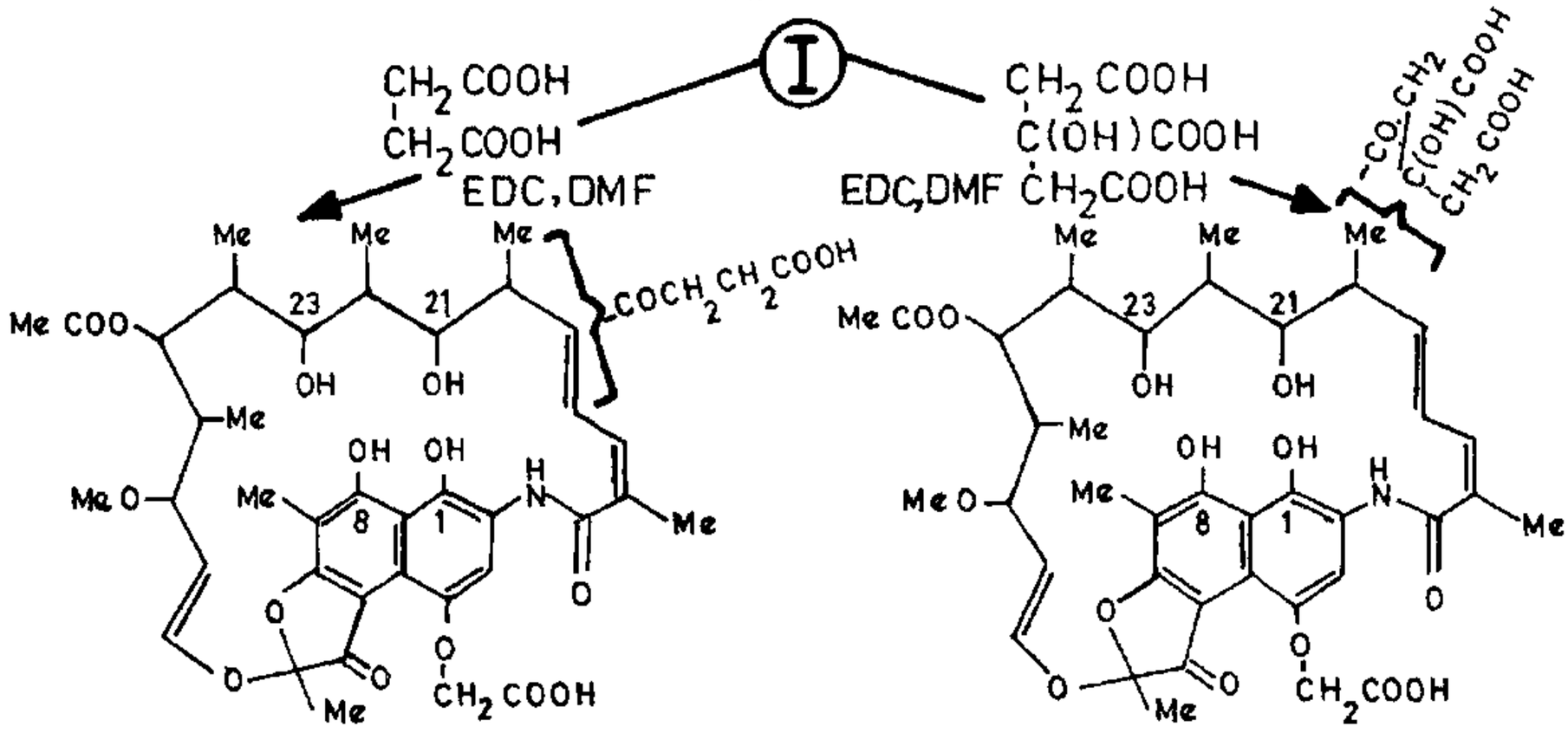
Synthesis of hemisuccinates and citrates of rifamycin B is shown in Scheme 1. To synthesize the succinate II, rifamycin B, I, (0.01 mmol) and succinic acid (0.1 mmol) were dissolved in 2 ml of dimethylformamide (DMF), and 1-ethyl-3-(3-dimethylaminopropyl)-carbamide (EDC) (Sigma, USA) (0.2 mmol) was added all at once and stirred for 1 h. The orange-red clear solution was allowed to stand at room temperature overnight. The mixture was lyophilized to a viscous deep blood-red oil and purified by TLC on silica gel using methanol: chloroform = 19:1 (v/v) as developing solvent.

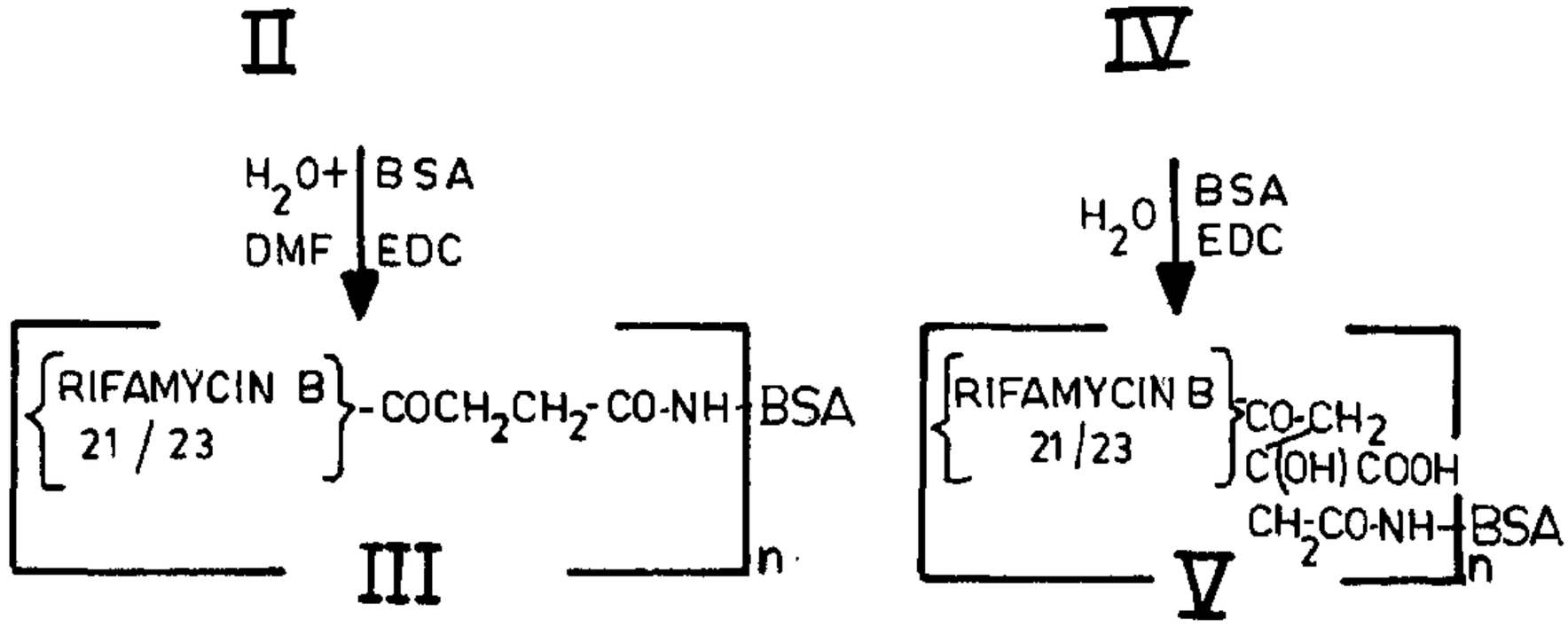
The citrate derivative IV was prepared from equimolecular amounts of citric acid and rifamycin B in presence of 5 times (molar ratio) of EDC in DMF following the procedure as above. TLC was carried out on silica gel and developed by methanol:chloroform = 4:1 (v/v).

A solution, 200 μl, of bovine serum albumin (BSA), (Sigma, USA) in water (0.1%) was added to the hemisuccinate solution, 1 ml, in DMF (5 μmol/ml). Water and DMF were added alternately, 10 μl each time, with constant shaking till the cloudiness decreased. EDC, 50 mg, was added all at once, the solution became clear, stirred for 4 h at room temperature to obtain a clear orange-red solution. It was then extensively dialysed (500 ml × 6) for two days and finally lyophilized to get III.

Rifamycin B citrate (IV) was coupled to BSA in two batches: In batch 1, a saturated solution of rifamycin B citrate in water (10 ml) was gently stirred and BSA,







20 mg, was added slowly and dissolved. EDC, 250 mg, was added to the stirring solution. The pH of the solution remained at 4.5 to 5.0 without adjustment. After stirring for 1 h at room temperature, the product was dialysed against water (1000 ml × 6) for 2 days in cold to obtain a clear golden ·yellow dialysate, and lyophilized to obtain V (batch 1).

In batch 2, a saturated solution of rifamycin B citrate in DMF was added dropwise slowly to a clear saturated aqueous solution (10 ml) of the compound with stirring till it just starts turning turbid. This near-turbid solution was coupled with BSA (50 mg) in presence of EDC (500 mg) as before and dialysed to obtain a deep orange-yellow slightly cloudy dialysate, and lyophilized to get V (batch 2).

Three adult rabbits (all males, Belgian strain), each weighing 1.5-2 kg, were used for immunization. The citrate conjugate V (batch 2) (1.2 mg protein weight) was emulsified by blending in 0.5 ml PBS and 0.5 ml of Freund's adjuvant and injected subdermally at multiple sites in each rabbit on day 0, 4, 8, 15 and 25. The first

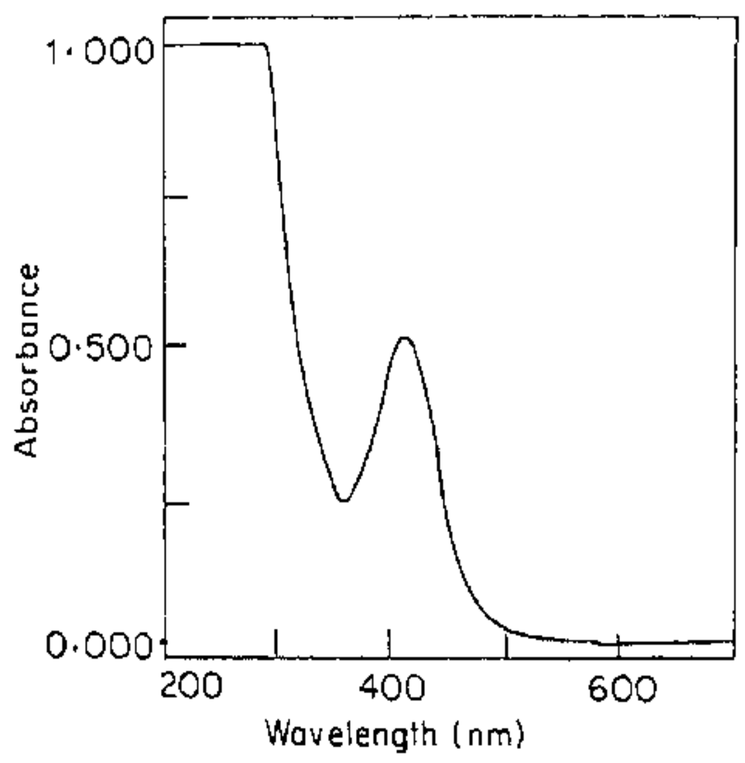


Figure 1. Absorption spectrum of rifamycin B (I).

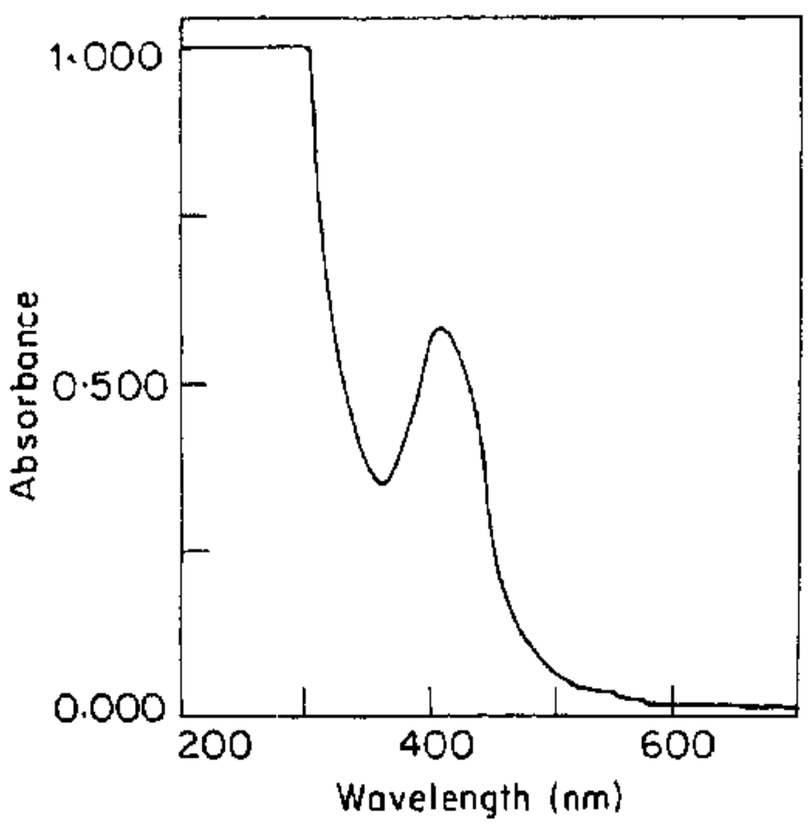


Figure 2. Absorption spectrum of the succinate derivative (II); (head band).

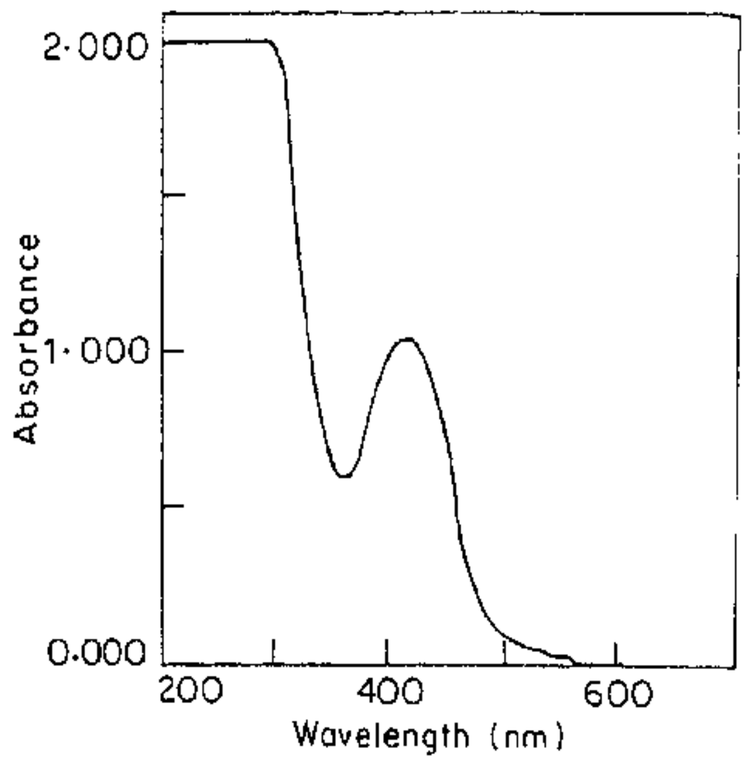


Figure 3. Absorption spectrum of the succinate derivative (II), (tail band).

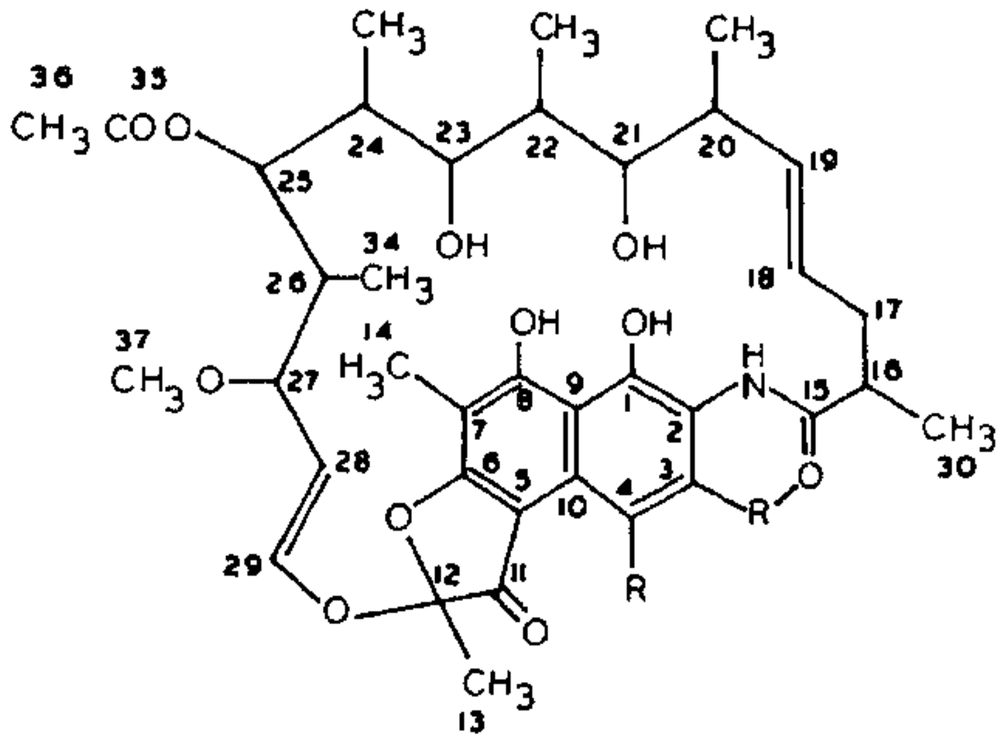
injection was given with complete and the rest with incomplete adjuvant. On day 35 serum was collected by ear vein puncture and tested for the presence of antibody by the Ouchterlony double diffusion method[12], after removing BSA-specific antibody[1,3]. To increase the antibody titre booster injections were given (up to seven injections) at 7-day intervals. The immunization with the succinate conjugate III was similarly carried out.

Rifamycin B (I), with low solubility in water (0.27%) and having many functional groups, is difficult to derivatize at a desired position by simple methodology under mild conditions. All these serious bottle-necks have been overcome by synthesizing compounds II–V (Scheme 1).

In the synthesis of the hemi-succinate (II) the two predominant products ($R_f = 0.48$, overlapping bands) were formed with only traces of another compound ($R_f = 0.62$), as shown by TLC. The starting material ($R_f = 0.91$) was completely absent, thus showing the reaction to be quantitative. Both the overlapping bands of II were separated by exhaustive preparative TLC, and two products (head : tail) were found in the ratio of 45 : 55, as measured from UV absorption.

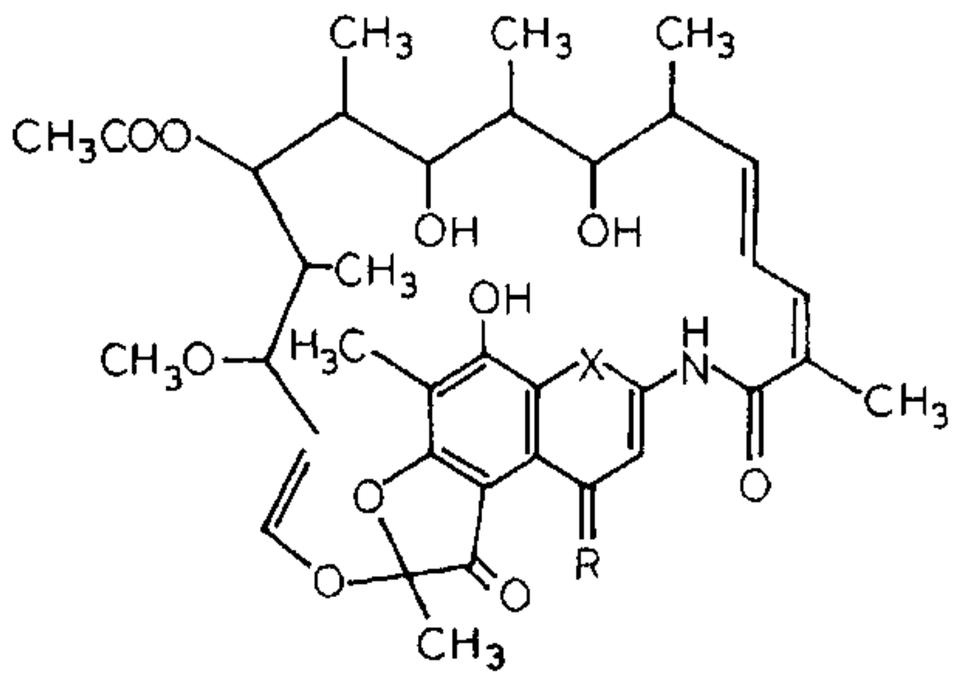
The absorption maxima (λ_{max}) of I, II (head band), II (tail band) were found to be 412.5, 412.5, and 415 nm (Figures 1–3) respectively in methanol. The absence of an appreciable change in the λ_{max} of the product II (both components) suggests that the sites of succinoylation are positions 21 and 23 or both. The two overlapping products probably indicate the esterification at both of these positions.

This particular derivatization at C_{21}/C_{23} of I has important implications[1,3,13]. Since the structural difference[11] between rifamycin B and all other ansamycins (Figures 4 and 5) is only at the aromatic nucleus, derivatization at C_{21}/C_{23} of I should facilitate the production of anti-rifamycin B antibody with little cross-reactivity with other related ansamycins.



Name	R	R'
Rifamycin SV	OH	H
Rifamycin L	$OCOCH_2OH$	H
3-Methylthiorifamycin SV	OH	SCH_3

Figure 4. Structural analogy of natural rifamycins.



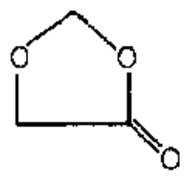
Name	R	X
Rifamycin S	O	C=O
Rifamycin O		C=O
Rifamycin G	O	O

Figure 5. Structural analogy of natural rifamycins.

The citrate derivative IV was similarly identified. Here also only traces of unreacted compound could be detected thus showing that this reaction was also quantitative. The two overlapping products appeared at $R_f = 0.59$ with only traces of another product observed between I and IV on TLC. That the derivatization in IV was at 21 and 23—positions were indicated as before.

Both the compounds II and III are insoluble in water, although II is moderately soluble in methanol and freely soluble in DMF. On the other hand, compounds IV and V derived from citrate are soluble in water, and the citrate derivative IV is soluble in methanol and freely soluble in DMF.

This difference in aquo-solubility is of fundamental importance. Since, rifamycin B (I) itself is sparingly soluble in water, its derivatives will normally be also insoluble in water. We have taken rigorous consideration and have chosen the derivatizing moiety on the following ground. It must have a number of hydrophilic functional groups a part of which can be easily coupled covalently under mild conditions. It is also desirable that the derivatizing agent should be cheap and easily available.

We have found that citric acid fulfils all the criteria of an ideal derivatizing agent. With its four-functional groups in such a small molecule, its tremendous hydrophilicity and solubility in water (64.3%), it can easily be coupled by one of the terminal carboxyl groups to an OH or NH_2 group of the insoluble compound, but still left with a number of groups to impart aquo-solubility of the derivative which in turn can be coupled to a macromolecule for biological purposes.

This is corroborated by the fact that the citrate of rifamycin B (IV) as well as the conjugate V is soluble in water.

Its biochemical implications can be guessed from the fact that whereas the succinate conjugate (III) did not give rise to any antibody even after three months of immunization, the citrate conjugate (V) gave rise to anti-rifamycin antibody (titre = 4) in all the rabbits after 35 days only.

The methodology is simple and fast and involves only two steps: (i) Derivatization of the insoluble compound with citric acid in an organic solvent like DMF, and (ii) Coupling of this water-soluble citrate derivative with the protein, which are easy enough to be carried out in a biochemical laboratory. The methodology should find general application provided the organic compound in question has an OH or NH_2 group.

1. Bhattacharyya, A., Madyastha, K. M., Bhattacharyya, P. K. and Devanandan, M. S., *Biochem. Biophys. Res. Commun.*, 1981, **98**, 520.
2. Bhattacharyya, P. K. and Bhattacharyya, A., *Biochem. Biophys. Res. Commun.*, 1981, **101**, 273.
3. Bhattacharyya, A., Madyastha, K. M., Bhattacharyya, P. K. and Devanandan, M. S., *Indian J. Biochem. Biophys.*, 1981, **18**, 171.
4. Bhattacharyya, A. and Bhattacharyya, P. K., *Indian J. Biochem. Biophys.*, 1981, **18**, 425.
5. Sharma, P. D., *J. Sci. Ind. Res.*, 1986, **45**, 534.
6. Tirrel, D. A., Donaruma, L. G. and Turek, A. B. (eds), *Macromolecules as Drugs and as Carriers for Biologically Active Materials*, New York Academy of Sciences, New York, 1985, p. 1.
7. Widder, K. J. and Green, R., *Methods Enzymol.*, 1985, **112**, 259.

8. Bhattacharyya, A. and Pal, B. C., *Nucleosides Nuocleotides*, 1986, **5**, 265.
9. Bhattacharyya, A., Mitra, S. and Pal, B. C., *Nucleosides Nucleotides*, 1984, **3**, 353.
10. Bhattacharyya, A. and Pal, B. C., *Indian J. Biochem. Biophys.*, 1986, **23**, 303.
11. Corcoran, J. W. (ed.), *Antibiotics*, Springer-Verlag, New York, 1981, vol. IV, p. 12.
12. Ouchterlony, O., *Progr. Allergy*, 1958, **5**, 1.
13. Miller, G. A., Clark, R. C. and Jessee, E. J., *Hybridoma*, 1986, **5**, 353.

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A bacteriophage lytic enzyme gene as a marker for gene transfer and expression

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The plasmid pBR322 bearing the lytic enzyme gene of colitis bacteriophage was injected into 10 sets of 10 onion bulbs each, at multiple points, and germinated for 7 days. The lytic enzyme was detected in the extracts of 0–60% of the bulbs in each set by turbidometric assay. This lytic enzyme gene can be used as a marker gene for gene transfer in plants and animals and in chimaeric gene constructs to study regulation of gene expression.

VARIOUS genes of bacterial and animal origin have been used as reporter genes and positive selection markers in gene transfer experiments. The most commonly used reporter genes include genes for chloramphenicol acetyltransferase (CAT)[1,2], neomycin phosphotransferase (NPT II)[3,4], *Escherichia coli* β-glucuronidase (GUS)[5] and firefly luciferase[6]. CAT and GUS genes are widely used though they are poor selective agents. The NPT II gene is a good selective agent but the assay of the enzyme is not easy. These genes are also used in chimaeric gene constructs to determine promoter strengths and gene expression[7,8]. We report here the use of a lytic enzyme gene from a virulent phage of *E. coli*, the colitis phage[9] as a marker for gene transfer and expression.

The lytic enzyme gene was mapped on a 2.3 kb *Bam*HI fragment of the phage DNA and cloned in the plasmid pBR322[10,11]. The gene was completely sequenced and the amino acid sequence was derived. This enzymic protein was of 18.05 kd and contained 163 amino acids. The enzyme was purified using the cloned gene and the characteristics studied[10]. The pBR322 and the recombinant plasmid DNAs were prepared according to the method of Brinboim and Doly[12]. They were dissolved in 10 mM Tris-HCl, pH 7.6 and 0.5 mM $MgCl_2$ (TM buffer) and 0.5 μl portions containing 0.1 μg of DNA were injected into the lower one-third portion of onion (*Allium cepa*) bulbs of 2 cm diameter at about 100 points using a 10 μl Hamilton syringe. This facilitated the flooding of the DNA into the meristematic regions of the bulb. The injected onions were kept at 22°C for 60 min and then transferred to glass vials containing TM buffer and ampicillin, tetracycline and streptomycin (2 μg/ml each) so that the base of the bulbs is dipped in the buffer solution and incubated at 22°C in a BOD incubator for 7 days. Three to four whorls of the onions which are essentially nutritive, were peeled off. The injected region of the fast growing bulb, along with the stem and roots, was washed thoroughly with water and ground with 1 ml of TM buffer at 4°C in a mortar with a pestle. The unbroken cells and cell debris were centrifuged at 15,000 *g* and the supernatant fraction was collected and assayed for the enzymic activity.

Sensitized *E. coli* B cells prepared according to the method of Salser *et al.*,[13] were used as the substrate. The *E. coli* B cells were grown to mid-log phase in M9 medium, pelleted at 7000 *g*, suspended in TM buffer previously saturated with chloroform, washed with TM buffer and suspended in the same buffer to have an absorbance of about 0.6 at 450 nm. The enzyme activity was monitored by the decrease in $A_{450\,nm}$ after the addition of the extract.

The plasmid pBR322 or recombinant DNA were injected in sets of 10 bulbs at a time and incubated for 7 days at 22 C. The extract was prepared from each bulb and assayed for the lytic enzyme. This was repeated at least 10 times at different periods during the course of a year. The enzymic activity was absent in the extracts of all sets of onions that received the pBR322 DNA. In the extracts of two sets of onions and few onions in the rest of the 8 sets, which received the recombinant DNA, there was no detectable enzymic activity. However, in the extracts from 8 sets of onions, the enzymic activity was detected in bulbs, the number of which varied from 1 to 6. The results from a typical set of experiments are shown in Figure 1. The extracts from the pBR322 injected onions did not show any lytic enzyme activity (line 1). The extracts from onions into which pBR322 DNA bearing the lytic enzyme gene was injected showed significant enzymic activity (lines 2–4). However, the activity varied from bulb to bulb and experiment to experiment. A unit of enzyme activity is expressed as the amount of enzyme causing a decrease of 0.1 at $A_{450\,nm}$ per min at 30°C (ref. 13) which corresponds to 10 ng of the enzymic protein[10]. The enzyme activities varied from 0.5 to 50 units per bulb and represent 0.05–5.0 μg of the enzyme protein. The enzyme in these extracts was inactive on *Micrococcus lysodeikticus* cells