STUDIES ON 6-ACETYLMETHYLENEPENICILLANIC ACID (Ro 15-1903)

III. RELATIONSHIP BETWEEN *IN VITRO* ACTIVITY AND CHEMICAL STABILITY

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To resolve discrepancies between its enzymological activity and its *in vitro* or *in vivo* activity, 6-acetylmethylenepenicillanic acid (Ro 15-1903), a potent β-lactamase inhibitor, was investigated for its chemical stability and its ability to penetrate the bacterial cell envelope. Although Ro 15-1903 was fairly stable in water or saline, it was found to be unstable in a rich medium, in mouse plasma and in human serum. Decomposition half-lives in Tryptic Soy Broth (TSB) and mouse plasma were determined by spectrometry to be 1.3 hours and 12 minutes respectively. These values were confirmed by a biochemical method for determination of Ro 15-1903. Furthermore, a large enhancement of the *in vitro* activity was noticed when the assay medium was changed from TSB to a synthetic medium in which Ro 15-1903 was more stable. The ampicillin-potentiating activity marginally increased if a permeability mutant harboring the R6K plasmid, which codes for TEM-1 β-lactmase production, was used instead of the wild-type strain. These results prove that the chemical instability of Ro 15-1903 is the main cause of its disproportionally low activity *in vitro* and *in vivo*. Ro 15-1903 is not nonspecifically inactivated by proteins, since it did not lose its activity after incubation with bovine serum albumin (50 mg/ml) for 2 hours at 37°C. It seems to react specifically with β-lactamase.

6-Acetylmethylenepenicillanic acid (Ro 15-1903) is one of the most active specific β-lactamase inhibitors known so far when examined at the subcellular level[1,2]. On the basis of IC_{50}-values, for instance, it showed 60- and 1,350-times greater activity against TEM-1 β-lactamase than clavulanic acid and sulbactam, respectively. Unfortunately the *in vitro* and *in vivo* findings did not correspond to these enzyme level activities[2]. Synergistic activity of Ro 15-1903 in combination with ampicillin was only slightly greater than that of sulbactam and less than that of clavulanic acid. Furthermore, Ro 15-1903 activity was reduced to the sulbactam level *in vivo*. This discrepancy had several possible explanations: 1) poor penetration, 2) chemical instability, 3) inferiority in kinetic parameters, *e.g.* high turnover and slow inactivation. Because 3 has now been ruled out[3,4], we began studying Ro 15-1903 with respect to 1 and 2. This paper shows that its chemical instability in a rich medium and in serum is the major reason for the discrepancy.

Materials and Methods

Microorganisms

Escherichia coli TEM-1 harboring the R6K plasmid was obtained from Prof. WIEDEMANN (Bonn) and stored at −70°C in the presence of 12.5% glycerol.

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Media and Other Materials

Tryptic Soy Broth (TSB) was a product of Difco. The synthetic medium A contained 0.1 M Na,K-phosphate buffer (pH 7.3), 0.02 M ammonium sulfate, 1 mM magnesium chloride, 2 μM ferric ion, 1% glucose and 0.05% Casamino Acids (vitamin free, Difco). Medium A' has the same components as medium A except for a reduced concentration of phosphate (5 mM). Mouse plasma was prepared from the blood of several mice by centrifugation in the presence of 3.8% citrate. Human serum was obtained from Difco as a lyophilized preparation. TEM-1 β-lactamase was purified from *E. coli* TEM-1 as described[8].

Spectrophotometry

A microcomputer-aided spectrophotometer (Uvicon 810, Kontron) with which serial overlay spectrograms could be obtained automatically, was used in all experiments. Difference spectra of Ro 15-1903 in solutions with a high background, such as serum and TSB, were recorded.

Treatment of Ro 15-1903 with Hydroxylamine and Sodium Hydroxide

A 2 M hydroxylamine solution (10 μl) was added to 1 ml of 100 μM Ro 15-1903 solution to give a final concentration of 20 mM hydroxylamine. The spectra were recorded by serial overlay at 37°C. For base treatment, 400 μM Ro 15-1903 was mixed with an equal volume of 1 N sodium hydroxide and the mixture was incubated at 37°C. Aliquots of 0.5 ml were periodically withdrawn and neutralized by the addition of 0.5 ml of 0.5 N hydrochloric acid before spectroscopic examination.

Biochemical Determination of Ro 15-1903 in TSB and Serum

The stability of Ro 15-1903 was checked by spectrometry as well as by a biochemical method. Thirty microliters of a relevant medium were mixed with an equal volume of 4 mM Ro 15-1903 dissolved in bi-distilled water and incubated at 37°C. At various time intervals, 10 μl samples were removed and poured into 990 μl of cold sodium phosphate buffer (100 mM). Then 10 μl of the diluted samples were added to 980 μl of prewarmed assay mixture containing 550 nmol of sodium benzylpenicillin in sodium phosphate buffer. The assay was initiated by the addition of 10 μl of 0.2 μM purified TEM-1 β-lactamases. The final concentration of Ro 15-1903 in the assay medium would be 0.2 μM if no decomposition occurred during the first incubation time, since Ro 15-1903 is stable in the assay mixture for at least a few hours. The decrease in A_{240} was observed over a period of 5 minutes in a water-jacketed cell holder at 37°C. The concentration of Ro 15-1903 was determined by measuring the degree of progressive inactivation of the TEM-1 β-lactamase as follows:

From the recorded reaction-curve, differential values with respect to time, *i.e.* the velocity (V) at each time, was calculated. A straight line passing through the origin was obtained by plotting ln (Vo/V) against time, where Vo is the initial velocity. This treatment seems applicable because the kinetic data fits the following scheme[8]:

$$\mathrm{S+E} \rightleftarrows \mathrm{ES} \xrightarrow{} \mathrm{E+P}$$

$$\mathrm{E+I} \xrightarrow{\mathrm{k}} \mathrm{EI^*}$$

where S=substrate (benzylpenicillin), E=enzyme (TEM-1 β-lactamase), ES=substrate-enzyme non-covalent complex, P=product, I=inhibitor (Ro 15-1903), EI*=inactivated enzyme. (S) and (I) are assumed to be constant:

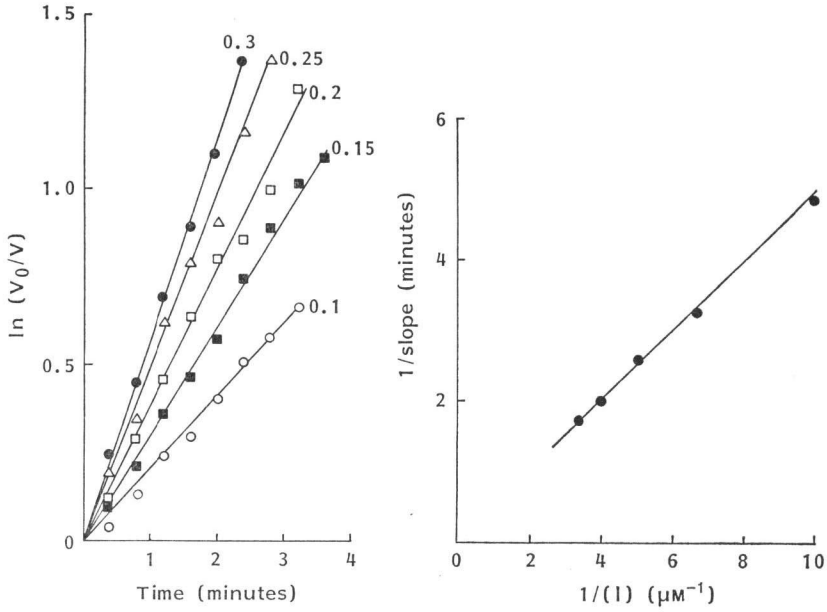
$$\ln (\mathrm{Vo/V}) = \frac{\mathrm{k}\, K_\mathrm{m}(\mathrm{I})}{K_\mathrm{m}+(\mathrm{S})}\mathrm{t} \qquad (1)$$

where K_m represents the Michaelis constant of benzylpenicillin for TEM-1 β-lactamase. Thus the slope of the plot of ln (Vo/V) against t is a function of (I) at constant (S). Equation 1 can be written in reciprocal form as

$$\frac{1}{\mathrm{Slope}} = \frac{1}{\mathrm{k}}\left(1+\frac{(\mathrm{S})}{K_\mathrm{m}}\right)\frac{1}{(\mathrm{I})} \qquad (2)$$

An example is illustrated in Fig. 1. This method is relatively simple to use, but not very accurate due to the inherent error of a reciprocal plot. The results obtained by this method, however, compared reasonably well with the results from the spectrophotometric assay, as shown in Results. An-

Fig. 1. Biochemical determination of Ro 15-1903.
Left: Plot of ln (Vo/V) against time. Ro 15-1903 concentration varied from 0.1 to 0.3 μM.
Right: Standard curve for Ro 15-1903 determination plotted from the graph on the left.



other disadvantage of this method is that a narrower range of Ro 15-1903 concentration (0.05~0.3 μM) can be tested. A more detailed description of the kinetic studies performed has been given previously[3].

In Vitro Assay

The potentiating activity of Ro 15-1903, clavulanic acid or sulbactam in combination with ampicillin was examined by the usual checkerboard titration method, diluting the reagents 2-fold serially on Microtest plates. The degree of superiority (N) of one inhibitor over the other was calculated from the equation:

$$N=2^{(a-b)/c}$$

where a is the total number of wells where no synergism between ampicillin and inhibitor A occurred (cells grow) and similarly, b is the total number of wells where no synergism between ampicillin and inhibitor B occurred. c is the number of ampicillin dilutions where potentiation was observed (c= constant for each comparison).

Results

Hydroxylamine and Base Treatment of Ro 15-1903

Ro 15-1903 dissolved in 100 mM sodium phosphate buffer (pH 7.0) showed two absorbance peaks in the UV-range, at 233 (ε 12,600) and 292 nm (2,200). Changes were observed with excess amounts of hydroxylamine and sodium hydroxide as shown in Fig. 2. In both cases absorbance at 233 nm decreased and a new chromophore was generated, although there was a difference in the peak wavelength, depending on the nucleophile used. This spectral change indicates that measuring the absorbance at 233 nm might give the concentration of Ro 15-1903, whatever the reactions may be.

Stability of Ro 15-1903 in TSB

Although Ro 15-1903 was quite stable in water or saline with half-lives of more than 2 weeks at

Fig. 2. Spectra of Ro 15-1903 (100 μM) before and after treatment with the nucleophiles NH_2OH or NaOH.

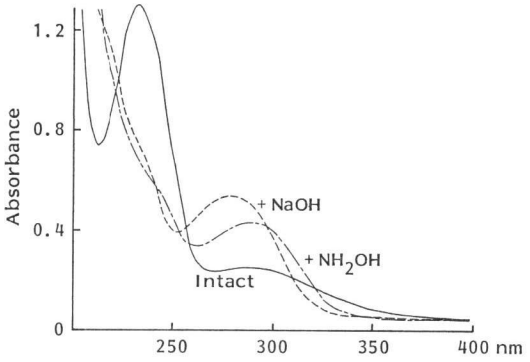
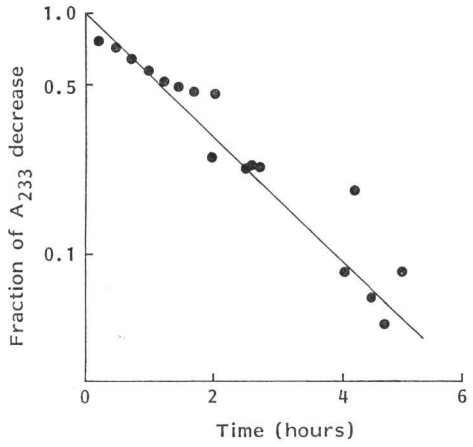


Fig. 3. Decomposition of Ro 15-1903 in TSB. Fraction of A_{233} decrease plotted logarithmically against time. Fraction of A_{233} decrease was calculated as $(A_0-A_t)/(A_0-A_h)$, where A_0 is the initial absorbance at 233 nm. A_t is the absorbance at time t and A_h the lowest absorbance obtained by hydroxylamine (20 mM) treatment.



room temperature, it was found to be unstable in TSB, the medium we had frequently been using for *in vitro* combination studies. Fig. 3 shows the change of absorbance at 233 nm with time. The absorbance exponentially decreased as a function of time, indicating a pseudo-first order reaction. Assuming that the absorbance at 233 nm would reach its minimal value after hydroxylamine treatment, which would correspond to the absorbance of completely decomposed Ro 15-1903, its half-life could be determined as 1.3 hours. Generation of a concomitant chromophore peak was not observed in TSB. The concentration of Ro 15-1903 in TSB was also determined by the biochemical method in order to confirm that the loss of inhibitory activity correlated with the spectral change. The half-life of 2 hours determined in this way agrees quite well with that determined by spectrometry, confirming the instability of Ro 15-1903 in TSB.

Improved Stability of Ro 15-1903 in Synthetic Medium

If chemical instability of Ro 15-1903 was the main cause of its disproportionally low activity *in vitro*, an increased activity should be detected by performing the combination experiments in a medium in which Ro 15-1903 is more stable. In synthetic medium A, Ro 15-1903 (50 μM) decomposed more slowly (half-life of 16 hours) than in TSB. This was regarded as insufficient for a bacteriological study where the incubation time is over 16 hours. By screening the effect of each component of medium A on the stability of Ro 15-1903, it was found that the phosphate buffer was the major inactivating agent. Decomposition was particularly fast in a solution more basic than pH 7.0 and at concentrations higher than 50 mM. Ro 15-1903 remained intact for longer at pH values less than 6 and at concentrations less than 10 mM. Finally we selected a modified medium A, designated medium A′, which contained 5 mM phosphate. The half-life of Ro 15-1903 in this medium varied with the concentration of Ro 15-1903; it was 22 hours at 2 mM and 40 hours at 100 μM. Other chemicals, like NaCl (0.5 M) and sodium citrate (1.9%), had no effect but 100 μM of Tris-buffer (pH 7.3) destroyed Ro 15-1903.

In Vitro Activity of Ro 15-1903

Checkerboard titration assays of Ro 15-1903 in combination with ampicillin were performed in

Fig. 4. Checkerboard titration of Ro 15-1903, clavulanic acid and sulbactam with ampicillin in complex and synthetic medium.

The solid line indicates the activity border in TSB, the dotted line the activity border in medium A′. The hatched part emphasizes the area where cell growth was noticed in TSB but not in medium A′. The test strain is *E. coli* TEM-1.

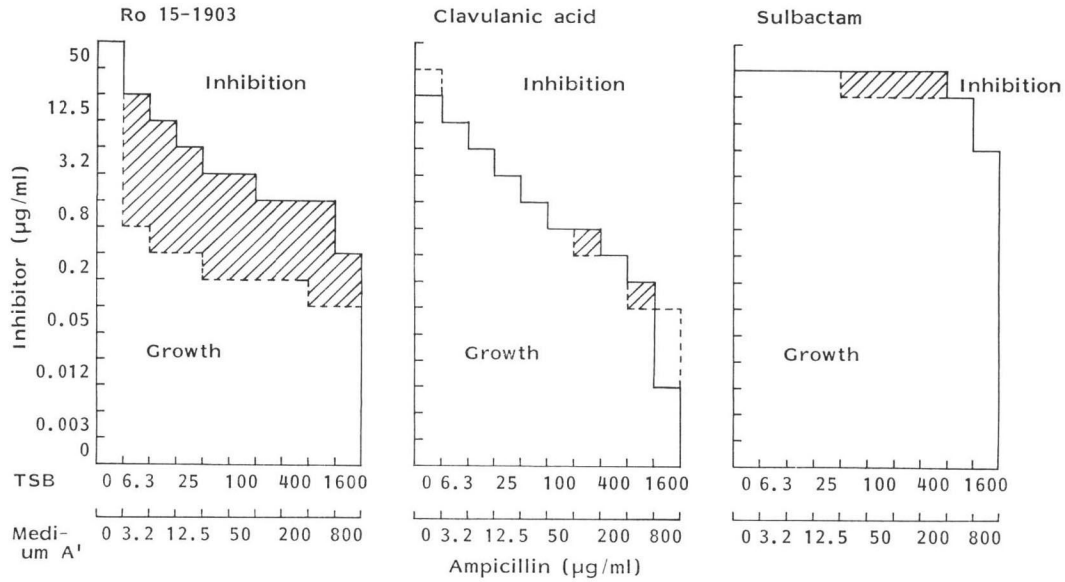


Table 1. Comparative synergistic activity of Ro 15-1903, clavulanic acid and sulbactam in combination with ampicillin, averaged from a range of ampicillin combinations*.

Medium	Ro 15-1903**	Clavulanic acid	Sulbactam
TSB	1	7.1	1/12
Medium A′	1	1/4.7	1/128

* Similar figures are obtained if the comparison is made by using those concentrations where optimal synergism occurred.

** The average potentiating activity of Ro 15-1903 was taken to be 1 in each medium. Degree of superiority calculated by $N=2^{(a-b)/c}$ as described under "Methods."

both TSB and medium A′ using *E. coli* TEM-1 as a tester strain. For comparison, clavulanic acid and sulbactam were tested in parallel. As shown in Fig. 4 susceptibility of *E. coli* TEM-1 to ampicillin differed in the two media, but the degree of synergism could be compared. Synergistic activity of Ro 15-1903 in combination with ampicillin was enhanced in medium A′, while the other two compounds were not affected by the medium used. An average 12-

Fig. 5. Decomposition of Ro 15-1903 (A) in mouse plasma; and (B) in a) bovine serum albumin (50 mg/ml), b) dialyzed human serum, c) human serum.

For explanation see Fig. 3.

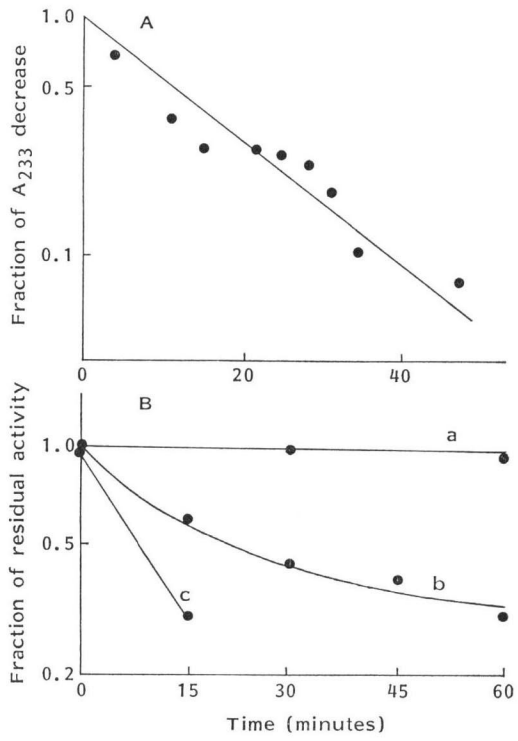


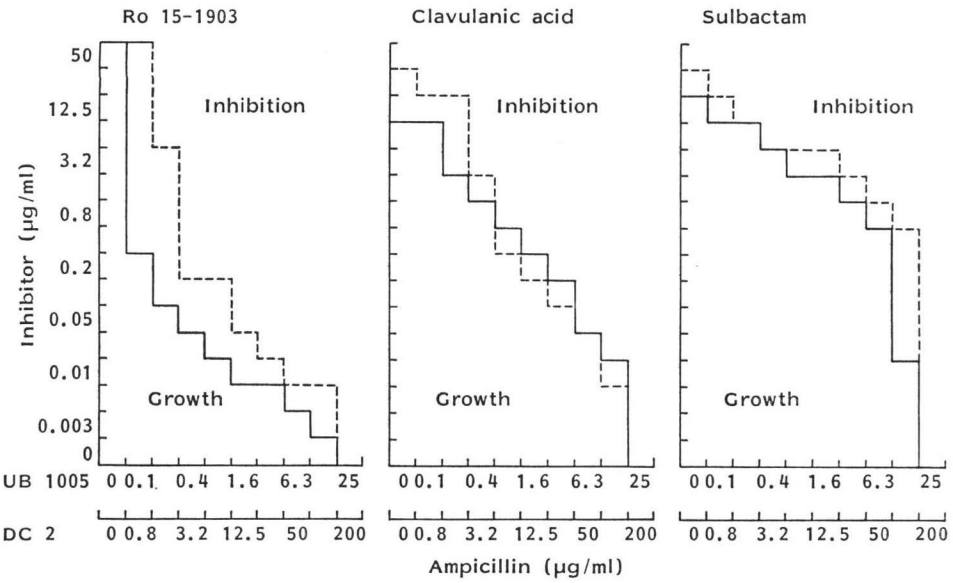
Table 2. Half-lives of Ro 15-1903 in different media.

Solvent	Ro 15-1903 concentration (μM)	Half-life[a] (hours) determined by	
		Spectrophotometry	Biochemical method
TSB	2,000	1.3	2
TSB (1/2 conc.)	1,000	2.2	—
Medium A	50	16	—
Medium A′	100	40	—
	2,000	22	
Mouse plasma (1/4 conc.)	2,000	0.2	0.2
Human serum (1/2 conc.)	2,000	—	0.15
Dialyzed human serum	2,000	—	30% remaining at 1 hour[b]
Bovine serum albumin (50 mg/ml)	2,000	—	Stable[c]

[a] All experiments were done at 37°C.
[b] Non pseudo-first order reaction.
[c] Inactivation was not detected within 2 hours.
— Not done.

Fig. 6. Checkerboard titration of β-lactamase inhibitors with ampicillin using permeability mutant, *E. coli* DC 2 (R6K), and its parent, *E. coli* UB 1005 (R6K).

The solid line indicates the activity boarder with the mutant, the dotted line the activity boarder with the wild type strain. Medium A′ was used.



fold difference in activity (calculated as described under "Methods") was observed. Consequently the relative activity of Ro 15-1903 compared to the other two inhibitors increased in the synthetic medium (Table 1).

Stability in Mouse Plasma and Human Serum

Information on the stability of Ro 15-1903 in serum also seemed necessary for resolving the discrepancy between the enzymatic data and the *in vivo* experiments. Mouse plasma diluted 1: 1 with 3.8% sodium citrate, and lyophilized human serum were used. Ro 15-1903 was unstable in both

liquids, decomposing with a half-life of about 10 minutes in mouse plasma at 37°C (Fig. 5a and Table 2). The spectrum of inactivated Ro 15-1903 was similar to that of NaOH-treated Ro 15-1903, producing a new chromophore. The stability of Ro 15-1903 in dialyzed human serum and in bovine serum albumin, the major protein in bovine serum, was also tested in order to know if Ro 15-1903 reacts non-specifically with proteins. As shown in Fig. 5b, Ro 15-1903 decomposed more slowly in dialyzed serum. The inactivation did not obey pseudo-first order kinetics. Bovine serum albumin (50 mg/ml) did not inactivate Ro 15-1903 within 2 hours at 37°C.

Comparative Potentiating Activity Evaluated with a Permeability Mutant

E. coli DC 2 is highly susceptible to various antibiotics and this has been attributed to a change of permeability in its outer membrane[5,6]. A set of strains harboring the R6K plasmid was constructed by conjugation between *E. coli* TEM-1 (R6K) as a donor and strain DC 2 or its parental strain, UB 1005, as recipient. As shown in Fig. 6, Ro 15-1903 exhibited 5.6-fold higher ampicillin-potentiating activity with strain DC 2 (R6K) than with strain UB 1005 (R6K), whereas the values for clavulanic acid and sulbactam were 1.1 and 2.3, respectively. This suggests that Ro 15-1903 penetrates somewhat less well than the two other inhibitors.

Discussion

As summarized in Table 2, Ro 15-1903 was labile in complex media and in biological fluids. In TSB it decomposed with a half-life of 1.3 hours at 37°C. This means that only 1% of the Ro 15-1903 added would remain intact in TSB after 8.5 hours incubation. Decomposition was much slower in medium A′ which contained low concentrations of phosphate. With this medium, the *in vitro* activity of Ro 15-1903 was enhanced. The effect of chemical stability was apparent in the checkerboard titration assay, when Ro 15-1903 became 5- and 130-fold more active than clavulanic acid and sulbactam, respectively. The enzyme assay and *in vitro* activity still differ by about a factor of 10. The reason for this is not known, but such a difference seems quite common.

The experiment with a penetration mutant indicated that penetration of Ro 15-1903 is worse than that of other related β-lactam compounds by a factor of 2~5, which, however, is not large enough to explain the disproportional activity between enzyme and *in vitro* assay. Therefore penetration is not a limiting factor for its *in vitro* activity.

The question of which components of the medium or serum inactivate Ro 15-1903 has not been answered. The compound was stable in NaCl (0.5 M), KCl (0.5 M), $(NH_4)_2SO_4$ (0.02 M), glucose (1%), $MgSO_4$ (1 mM) and sodium citrate (1.9%) but unstable in Tris-HCl buffer (100 mM, pH 7.3) and phosphate buffer (100 mM, pH 7.3). Its increased stability in dialyzed human serum and failure of the reaction to obey pseudo first order kinetics suggests a shortage of nucleophiles after dialysis to attack Ro 15-1903. Furthermore, Ro 15-1903 was not inactivated in bovine serum albumin (50 mg/ml) solution. These data, together with observations that Ro 15-1903 has almost no antibacterial activity and very little affinity for penicillin binding proteins of *E. coli* suggest a high specificity for β-lactamases. This view is supported by its kinetic and biological properties[2,3,4].

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