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CHEMISTRY OF TERRAMYCIN

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Terramycin, the newest of the broad spectrum antibiotics, is produced as an elaboration product of a recently discovered actinomycete, *Streptomyces rimosus*, which was so named because of the cracked appearance of its growth on the surface of solid media.[1, 9] The discovery of terramycin resulted from an intensive soil-screening programme carried out over a period of several years. The organisms were isolated [4] in pure culture and tested for their ability to inhibit the growth of pathogenic micro-organisms.

Recent communications have emphasized the broad scope of antimicrobial activity of terramycin. The list of inhibited pathogenic micro-organisms [2] includes many of the Gram-negative and Gram-positive bacteria (both aerobic and anaerobic), various rickettsiae, psittacosis virus, spirochetes, and certain other viral and protozoan organisms.

Terramycin may be isolated from broth filtrates of *Streptomyces rimosus* by a number of methods.[7] Since it is an amphoteric compound, forming salts with strong acids and bases, practicable methods of recovery include solvent extraction and precipitation of mixed inorganic salts.[8] In the initial isolation, a culture filtrate was extracted with *n*-butanol and transferred into dilute acid to give a crude concentrate of the antibiotic. Chromatography on Florisil — a magnesia-silica-gel adsorbent — yielded a high potency fraction, which was purified further by extraction into butanol and re-extraction into dilute acid. Pure crystalline terramycin dihydrate was obtained by dissolving the crude material in dilute acid, precipitating by neutralizing with alkali, and repeating this procedure.[7]

Paper partition chromatography, using *n*-butanol/acetic-acid/water and ascending solvent flow, was helpful in following the progress of purification. Under ultra-violet light, terramycin exhibits a bright yellow fluorescence, which property was used to detect it on the papergrams. In addition, the paper chromatograms were examined for antibiotic activity by locating the zones of inhibition after pressing them on agar-plates seeded with *Bacillus subtilis*.

Homogeneity was tested by countercurrent distribution between *n*-butanol and Clark & Lubs pH 2.5 buffer. Under these conditions, terramycin has a distribution coefficient of about 1.0, which is independent of concentration. The experimental data conformed closely to a theoretical curve for a single component.

The solubility method was employed to establish with greater certainty the high degree of purity of isolated terramycin hydrochloride.[8] Methanol was selected as the solvent for these studies, since at 35°C solutions of terramycin hydrochloride were found to be stable for long periods. Equilibrium solubility required approximately 18-20 hours for attainment. Calculations from solubility curves showed that a sample of twice-crystallized terramycin was not less than 99.5% pure.

In the course of the early work on terramycin, pure samples were subjected to a large number of stability tests under varying conditions. It was observed that terramycin could be heated at 100°C in vacuo for four days without any apparent loss in potency. In addition, dilute solutions at pH 1.0-9.0 showed no detectable losses on storage at 5°C for at least one month.

Terramycin is a pale-yellow compound having a composition best represented by the formula $C_{22}H_{24}N_2O_9$. It crystallizes readily from water as the dihydrate, which loses its water of crystallization on heating in vacuo at 100°C. The anhydrous compound melts at 184.5°-185.5°C, with decomposition. The antibiotic is amphoteric and forms well-defined salts with mineral acids and bases. Calculations based on titration data of terramycin hydrochloride in aqueous solution at 28°C gave *pKa'* 3.5, 7.6, 9.2. Approximately the same values are obtained from titration data for solutions in methanol-water mixtures in which terramycin is more soluble than in water. Among the acid salts of terramycin, the hydrochloride and the hydrobromide are the best characterized as yet. These are bright-yellow, beautifully crystalline compounds having the compositions $C_{22}H_{24}N_2O_9$,HCl and $C_{22}H_{24}N_2O_9$,HBr, respectively. The hydrohalides dissolve readily in water, but unless excess acid is added to a pH below 1.5, the crystalline free-base separates as the dihydrate on standing.

The ultra-violet spectra of terramycin and terramycin hydrochloride in methanol solution are quite similar : with peaks for terramycin at 270 mμ, log. $E = 4.27$, and at 370 mμ, log $E = 4.25$; and for terramycin hydrochloride at 267 mμ, log $E = 4.24$, and at 365 mμ, log $E = 4.17$. Addition of excess acid shifts both wave-length and extinction of the longer wave-length peak to somewhat lower values, while excess alkali shifts both wave-length and extinction to slightly higher values. The extinction of the shorter wave-length peak is enhanced by addition of either acid or alkali without much change in wave-length.

The disodium and dipotassium salts of terramycin, having the compositions $C_{22}H_{22}N_2O_9Na_2$, $2H_2O$ and $C_{22}H_{22}N_2O_9K_2$, $2H_2O$, respectively, are

yellow, crystalline hydrates which are readily soluble in water and relatively insoluble in ethanol. Freshly prepared aqueous solutions are bright yellow, turning darker on standing. They lose very little biological potency over a period of four days when held at temperatures below 15°C. The calcium and magnesium salts of terramycin are only slightly soluble in water. Terramycin readily forms mixed salts with a number of pairs of bivalent metal ions. The barium-calcium and barium-magnesium salts are very insoluble in water and precipitate readily from aqueous solutions at pH 8.5 to 9.5.

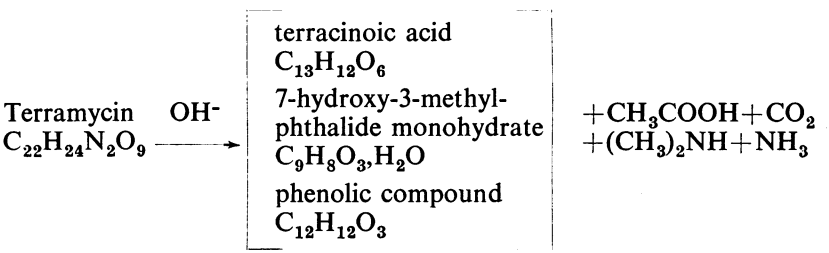
The low solubilities of the mixed salts provide convenient methods of isolating the antibiotic from fermentation broths. For example, the mixed barium-calcium salt of terramycin can be precipitated from filtered culture broth. After filtering, the antibiotic is liberated from the impure mixed salt with excess sulfuric acid, filtered, precipitated by neutralization, dissolved in methanol containing calcium chloride, and crystallized as the hydrochloride by the addition of excess hydrochloric acid. The impure hydrochloride can be purified further by converting to the base, dissolving in methanol containing calcium chloride, and crystallizing as the hydrochloride by the addition of hydrochloric acid.

In this method, the calcium chloride is added to the methanol to take advantage of the marked tendency of terramycin to form complexes with inorganic salts. The complex-forming property of terramycin with calcium chloride, cupric chloride, etc., was measured by a qualitative titration method based on the increase in acidity as a function of complex formation. The titration curves of terramycin, containing calcium chloride, are characterized by a sharp initial rise in pH on the addition of one equivalent of sodium hydroxide per mole of terramycin. In all probability, several complexes exist in solution, and from the titration curve it can be seen that there is good evidence for the formation of $(\text{terramycin})_4\text{CaCl}_2$, and $(\text{terramycin})_2\text{CaCl}_2$. Under one set of conditions, a crystalline complex has been isolated having a composition in fairly good agreement with the formula $(\text{terramycin})_4\text{CaCl}_2$. To be noted also is the strong inflexion-point in the titration curve which occurs in the vicinity of three equivalents of base.

Terramycin has been subjected to a large number of standard diagnostic tests. Some of these are difficult to interpret, but there is adequate evidence for the phenolic character of the antibiotic. A modified nitro-chromic acid Fearon-Mitchell test with terramycin is indicative of an oxidation-reduction system analogous to that of a quinone-hydroquinone configuration. In addition, terramycin gives positive tests with Fehling, Molisch, ferric chloride, Friedel-Crafts', and aminoantipyrine reagents, a deep-red colour with sulfuric acid and sodium nitrite, and a deep-red colour with diazotized β-naphthylamine. It gives negative tests with carbonyl reagents and a negative furfural test. On hypo-iodite oxidation, terramycin consumes about seventeen equivalents of iodine yielding, among other products, acetic acid, two moles of iodoform and one mole of dimethylamine per mole

of terramycin. The antibiotic forms a triacetyl derivative and a crystalline pentabromo derivative. By the Zerewitinoff method, using methyl magnesium bromide in pyridine, anhydrous terramycin base shows 7 to 8 active hydrogen atoms per mole.

Terramycin is readily degraded by the action of aqueous alkali.[6] On boiling a 20% aqueous sodium hydroxide solution of terramycin, one mole each of ammonia and dimethylamine are evolved within 24 hours. When the hydrolysis is carried out in the presence of zinc, a number of crystalline products can be isolated. The major product, isolated in 50% yield as a white, crystalline compound, has been named terracinoic acid (melting-point 232°-234°C, with decomposition).



The compound is optically inactive and has a molecular formula $C_{13}H_{12}O_6$. Among the products isolated in relatively low yield from this reaction mixture are a colourless, crystalline phenolic lactone, 7-hydroxy-3-methyl-phthalide,[3] $C_9H_8O_3,H_2O$ (melting-point 110°-112°C), and a colourless, monoacidic phenolic compound, $C_{12}H_{12}O_3$ (melting-point 169°-170°C). Acetic acid and carbon dioxide are also produced in this alkaline degradation.

Terracinoic acid[5] is a tribasic acid having *pKa'* values 2.6, 4.7, and 9.5. Three active hydrogens are found by Zerewitinoff's procedure and the expected mono-, di-, and tri-metal salts can be formed. The existence of a carboxylic-acid group in terracinoic acid is established by a smooth acid catalysed or thermal decarboxylation to yield a colourless, crystalline compound, decarboxyterracinoic acid, $C_{12}H_{12}O_4$ (melting-point 169°-170°C). The formation of a crystalline oxime indicates the presence of a carbonyl group in terracinoic acid. The presence of a benzene ring in terracinoic acid is demonstrated by the formation of trinitrohydroxybenzoic acid by nitric-acid oxidation.

When terramycin is fused with alkali at 200°C, appreciable quantities of salicylic acid (11%), *m*-hydroxybenzoic acid (5%), and succinic acid (17%) are isolated from the volatile acidic fraction.

Under controlled acid conditions, terramycin is cleaved smoothly to yield several crystalline biologically inactive derivatives. The series of acid

reactions is initiated by the slow rearrangement of terramycin in two equivalents of 1.5 N hydrochloric acid at 60°C to yield a yellow, crystalline hydrochloride, $C_{22}H_{24}N_2O_9$,HCl (melting-point 198°-202°C, with decomposition), with *pKa'* 3.1, 4.7, and 8.0. The free base is a stronger acid than terramycin. This rearrangement product of terramycin is optically active: $[\alpha]_D^{25°} + 185°$. Under the same conditions, terramycin shows $[\alpha]_D^{25°} - 197.0°$. Like terramycin, the rearrangement product is amphoteric, fluorescent under ultra-violet light, and complex-forming with calcium chloride. Interestingly enough, treatment with alkali and zinc does not produce terracinoic acid from the isomer. Qualitatively, the rearranged product behaves somewhat like terramycin : it gives positive Fehling and Pauly tests and a deep emerald-green colour with methanolic ferric chloride. It gives also a positive aminoantipyrine test for phenols. On heating the isomer at 60°C in a mixture of pyridine and acetic anhydride, it forms a nicely crystalline acetyl derivative which gives a negative ferric chloride test.

On longer contact with hydrochloric acid, the rearranged product splits out dimethylamine and yields an optically active compound $C_{20}H_{15}NO_8$ (melting-point 210°-213°C). The compound is soluble in most organic solvents, in sodium bicarbonate, and in ammonia. It gives a deep-green colour with methanolic ferric chloride, slowly absorbs bromine, gives a light-green colour with Friedel-Crafts' reagent and a yellow colour with concentrated sulfuric acid. On refluxing the compound in 20% alkali, the nitrogen is eliminated as ammonia. With anhydrous sodium acetate and acetic anhydride at 100°C, a crystalline acetyl derivative is obtained which melts sharply at 229°C and shows a negative ferric chloride test.

Treatment of terramycin with strong acids almost immediately gives an insoluble red product. However, when the $C_{20}H_{15}NO_8$ compound is heated in 12 N acids, ammonia and carbon dioxide are liberated and a new fragment is obtained. The compound is sensitive to air, alkali, and permanganate, gives colour tests characteristic of phenols, and is optically inactive. The compound also forms a crystalline acetyl derivative by heating in acetic anhydride and sodium acetate. The acetyl derivative is very resistant to permanganate oxidation.

An interesting aspect of the acid degradation fragments is that the comparison of the absorption spectra of the acid series with their acetyl derivatives shows that there is a definite hypsochromic shift of the major peaks in the acetyl compounds.

Various approaches used in the degradation studies have led to certain alterations in the terramycin molecule. As a consequence, each of the fragments presents a distinct problem. Work is in progress to relate these fragments to the structure of terramycin and this will be reported as certain structural features are resolved.

SUMMARY

Terramycin, the newest of the broad spectrum antibiotics, is produced as an elaboration product of a recently discovered actinomycete, *Streptomyces rimosus*, isolated from soil. Recent communications have emphasized the strong antimicrobial activity of terramycin against a wide variety of micro-organisms. Terramycin may be isolated and purified by several methods. Its high purity has been demonstrated by solubility measurements, countercurrent distribution, and paper chromatography.

Terramycin is a pale-yellow compound having a composition best represented by the formula $C_{22}H_{24}N_2O_9$. The antibiotic is amphoteric and forms well-defined salts with mineral acids and bases. The hydrated amphoteric base and hydrohalides of terramycin have been characterized. Calculations based on titration data of terramycin hydrochloride in aqueous solution gave *pKa'* 3.5, 7.6, and 9.2. The disodium and dipotassium salts of terramycin are yellow, crystalline hydrates which are readily soluble in water and insoluble in alcohol. The calcium and magnesium salts are only slightly soluble in water, and mixed salts of bivalent metals precipitate readily from aqueous solutions. Terramycin forms complexes with metal salts and the acidity of terramycin is greatly increased in certain of these complexes.

Terramycin is degraded by the action of hot alkali to yield ammonia, dimethylamine, carbon dioxide, acetic acid, terracinoic acid, 7-hydroxy-3-methylphthalide, and the phenolic compound $C_{12}H_{12}O_3$. Under controlled acid conditions, terramycin is cleaved smoothly to several crystalline biologically inactive derivatives. Acid degradation is initiated by the slow rearrangement of the terramycin molecule, followed by loss of water and dime thylamine, to yield an optically active compound, $C_{20}H_{15}NO_8$. The latter loses ammonia and carbon dioxide on treat-

RÉSUMÉ

La terramycine, le plus récent des antibiotiques à spectre d'activité étendu, est une substance élaborée par un actinomycète récemment découvert et isolé du sol, *Streptomyces rimosus*. Des communications récentes ont mis en évidence l'action puissante de cet antibiotique sur des micro-organismes très variés. La terramycine peut être isolée et purifiée par différentes méthodes. Son degré de pureté élevé a été prouvé par des mesures de solubilité, la distribution à contre-courant et la chromatographie sur papier.

La terramycine est un composé jaune pâle, dont la composition correspond à la formule $C_{22}H_{24}N_2O_9$. Cet antibiotique est amphotère et forme des sels bien définis avec les acides minéraux et les bases. La base amphotère hydratée et les hydrates halogénés de la terramycine ont été caractérisés. Les calculs basés sur la titration du chlorhydrate de terramycine en solution aqueuse ont donné pour *pKa'* les valeurs de 3,5; 7,6; 9,2. Les sels disodique et dipotassique de la terramycine sont des hydrates cristallins jaunes, facilement solubles dans l'eau et insolubles dans l'alcool. Les sels de calcium et de magnésium ne sont que légèrement solubles dans l'eau, et un mélange de sels de métaux bivalents précipite facilement de solutions aqueuses. La terramycine forme des complexes avec les sels métalliques et son acidité est fortement accrue dans certains de ces complexes.

La terramycine est décomposée par l'action des alcalis à chaud; il se forme de l'ammoniaque, de la diméthylamine, du gaz carbonique, de l'acide acétique, de l'acide terracinoïque, de l'hydroxy-7 méthyl-3phtalide, et le composé phénolique $C_{12}H_{12}O_3$. En milieu acide ménagé, la terramycine est scindée progressivement en dérivés cristallins biologiquement inactifs. La décomposition en milieu acide commence par un réarrangement au sein de la molécule de terramycine, suivi d'une perte d'eau et de diméthylamine, aboutissant à la formation d'un composé

ment with 12 N acids to yield a nitrogen-free hydroxy derivative.

optiquement actif, $C_{20}H_{15}NO_8$. Ce dernier, en perdant de l'ammoniaque et du gaz carbonique, par traitement au moyen d'acides 12 N, donne un dérivé hydroxy, non azoté.

REFERENCES

1. Finlay, A. C., Hobby, G. L., P'an, S. Y., Regna, P. P., Routien, J. B., Seeley, D. B., Shull, G. M., Sobin, B. A., Solomons, I. A., Vinson, J. W. & Kane J. H. (1950) *Science*, **111**, 85
2. Hobby, G. L., Dougherty, N., Lenert, T. F., Hudders, E. & Kiseluk, M. (1950) *Proc. Soc. exp. Biol., N.Y.* **73**, 503
3. Hochstein, F. A. & Pasternack, R. (1951) *J. Amer. chem. Soc.* **73**, 5038
4. Kane, J. H., Finlay, A. C. & Sobin, B. A. (1950) *Ann. N.Y. Acad. Sci.* **53**, 226
5. Pasternack, R., Bavley, A., Hess, G. B., Conover, L. H., Brunings, K. J. & Hochstein, F.A. (1951) *Alkaline degradation of terramycin* (Paper presented to 12th International Congress of Pure and Applied Chemistry, New York ; see also *Abstr. Pap. Int. Congr. pure appl. Chem.* 1951, p. 281)
6. Pasternack, R., Regna, P. P., Wagner, R. L., Bavley, A., Hochstein, F. A., Gordon, P. N. & Brunings, K. J. (1951) *J. Amer. chem. Soc.* **73**, 2400
7. Regna, P. P. & Solomons, I. A. (1950) *Ann. N.Y. Acad. Sci.* **53**, 229
8. Regna, P. P., Solomons, I. A., Murai, K., Timreck, A. E., Brunings, K. J. & Lazier, W. A. (1951) *J. Amer. chem. Soc.* **73**, 4211
9. Sobin, B. A., Finlay, A. C. & Kane, J. H. (1950) US Pat. No. 2,516,080. 18 July 1950. *Terramycin and its production* (Summarized in *Off. Gaz. U.S. Pat. Off.* **636**, No. 3)