Metabolic Study of 2-(Acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline in Chickens

Detection of an Active Metabolite, 5,6-Dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole

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After oral treatment of chickens with antazonite (R 6438), six basic products have been found by thin-layer chromatography in extracts of feces, one of them being the parent compound. The most important metabolite (metabolite No. 3) was isolated by column chromatography, and its structure was elucidated by physicochemical methods and confirmed by synthesis and analysis. The new product, 5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole (antienite, R 8141) was about 4 times as active as the original compound. There is some evidence suggesting that the anthelmintic activity of R 6438 is due to this metabolite. Two other metabolites were identified by comparison with existing substances on thin-layer chromatoplates. Further synthetic and parasitological work has shown that chemical modification of R 8141 may result in still more active substances. The most interesting analog, 2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-*b*]thiazole (tetramisole, R 8299) has been chosen for further detailed parasitological and pharmacological investigation.

THIENPONT and co-workers (1, 2) assigned good anthelmintic activity to 2-(acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline (R 6438, antazonite). When given orally to chickens in a dose of 160 mg./Kg. body weight, all *Ascaridia* sp., all *Heterakis* sp., and about 30% *Capillaria* sp. are expelled within 48 hr.

In order to gather information about the metabolism of R 6438, serum, different organs, eggs, and the feces of hens treated with the product were analyzed. The samples were homogenized, extracted by conventional techniques, and the extracts were analyzed by thin-layer chromatography (TLC) on Silica Gel G. A modified Liebermann-Burchard reagent was used as a spray to reveal the spots.

In the feces of chickens, at least five metabolites of antazonite occurred, together with the parent compound. Metabolite No. 3 was found in the greatest concentration, accounting for about 20% of the administered dose. The sum of the other metabolites was less than 5% of the dose of R 6438 given.

To elucidate the structure of the different metabolites, a total amount of 6 Gm. of R 6438 was given to 8 chickens, each animal receiving 750 mg., divided in three oral portions of 250 mg. The doses were administered at intervals of 32 and 38 hr. The feces (2.5 Kg.), collected over a period of 4.5 days after the first treatment, were dried, homogenized, and suspended in dilute acid. The basic compounds were extracted and purified by liquid–liquid extraction, and the final solution was concentrated to a small volume. This concentrate was used for thin-layer and for column chromatography on silica gel.

The fractions of eluate containing the major metabolite were combined and concentrated. Small samples of it were used for measurement of ultraviolet and infrared spectra. Both absorption patterns suggested a structure containing a 2-substituted, nonconjugated thienyl ring, and a C=N double bond in a five-membered ring. It was presumed at that moment that the product could be 5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole. The remainder of the concentrate was treated with oxalic acid yielding an oxalate salt, which, after recrystallization, gave 190 mg. of a crystalline product. This salt was analyzed (basic equivalent, C, H, N, and S determination, U. V. and I. R. spectra, TLC) and shown to be $C_9H_8N_2S_2.C_2H_2O_4$. TLC showed that it was a pure product, whereas both spectra and analyses were in agreement with the proposed formula. Treatment of one hen with 100 mg. of the material (corresponding to 70 mg. of the base) showed that the metabolite possessed enhanced anthelmintic properties (1).

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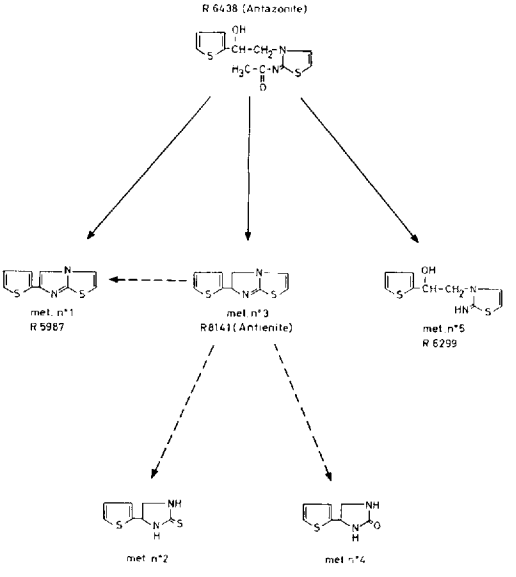
5,6 - Dihydro - 6 - (2 - thienyl) - imidazo[2,1 - *b*]-thiazole was prepared synthetically (3); the melting point of its oxalate salt and mixed melting point with the oxalate of the metabolite isolated from the feces proved the identity of the structures. TLC patterns, ultraviolet, and infrared spectra of both oxalates were also identical.

Two other metabolites were identified by comparison of their TLC patterns with those of known products related to R 6438. One of them, designated as metabolite No. 1, is an unsaturated analog of metabolite No. 3 and is 6-(2-thienyl)-imidazo[2,1-*b*]thiazole (R 5987) (3). The other identified product, designated as metabolite No. 5, is the deacetylated derivative of R 6438 and is 2-amino-3[-2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline (R 6299) (3). Both metabolites are practically devoid of anthelmintic properties (1). The remaining two metabolites (No. 2 and 4) could not be identified with certainty. However, it is supposed that both metabolites are degradation products of metabolite No. 3. This is supported by the fact that an analogous breakdown has been observed during metabolic studies on 5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole (R 8193), which is strongly related to R 8141. The structure of the metabolite No. 2 would then be 2-thio-5-(2-thienyl)-imidazolidine, whereas No. 4 would correspond to 2-oxo-5-(2-thienyl)-imidazolidine.

It is supposed that the anthelmintic activity of R 6438 is due to its major metabolite, R 8141. This is supported by the following facts.

(*a*) R 6438 is active as an anthelmintic in poultry, but not in rats (1). Its metabolite, R 8141, is active in both species. This metabolite occurs in feces of chickens and pigeons, but not in feces of rats. (*b*) About one-fifth of the R 6438 administered orally to chickens is found as R 8141 in feces. This metabolite is about 4 times as active as R 6438.

The metabolic degradation of R 6438 is represented in Scheme I.



Scheme I

TABLE I.—SOLUBILITY OF R 6438 AT ROOM TEMPERATURE

Solvent	pH	Solubility of R 6438, %
Water	6.4	0.065
0.01 *N* Hydrochloric acid	3.0	0.3
0.1 *N* Hydrochloric acid	2.1	2.5
Hexane	...	0.08
Chloroform	...	9.6
Methyl alcohol	...	6.9
Ethyl alcohol	...	4.1
Isopropyl alcohol	...	1.8
Diethyl ether	...	0.5
Acetone	...	5.4
Methyl isobutyl ketone	...	1.6
Ethyl acetate	...	2.4

TABLE II.—FECAL COLLECTIONS FOLLOWING TREATMENT

After First Treatment, hr.	Wt. of Feces, Gm. Fresh	Dried	Dry Material, %
32	1028	211	21.5
60	648	147	22.7
84	424	91	21.5
108	449	83	18.5
Total	2549	532	20.9

EXPERIMENTAL, RESULTS, AND DISCUSSION

Physicochemical Properties of Antazonite.—Antazonite, 2-(acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline, has the structure as shown in Scheme I. It was synthetized by Raeymaekers (3). It occurs as a yellowish to brown-white, fine powder without odor or taste, with a melting point between 131 and 134°. The solubility of antazonite in water increases with decreasing pH (Table I). It is soluble in chloroform, methyl alcohol, ethyl alcohol, and acetone, sparingly soluble in isopropyl alcohol and methyl isobutyl ketone, and slightly soluble in diethyl ether.

The change in U.V. absorption upon variation in pH of the solutions indicates that antazonite has a pK value of about 3.6.

Principles of Extraction of R 6438.—The extraction of R 6438 is based on the fact that the compound forms a water-soluble salt, the solubility of which increases with decreasing pH. When the aqueous solution is made alkaline, antazonite precipitates, so the free base can be transferred into a water-immiscible organic solvent, such as ether, chloroform, or benzene.

Based on these principles, the first experiments were carried out on eggs of chickens, which were treated orally with one dose of 160 mg. R 6438/Kg. body weight. After extraction of these eggs and TLC of the extracts, four spots were found. The total amount of these different compounds was about 90–260 mcg./egg, within 48 hr. In order to elucidate the structure of these unknown products, further work was done on the feces of chickens.

Treatment of the Hens and Collection of Feces.—Eight white leghorn chickens, maintained each in a cage were given antazonite in No. 00 gelatin capsules, with invervals of 32 and 38 hr. Each bird was given 750 mg. of R 6438 and 600 mg. of sodium bicarbonate. They were given water and food *ad libitum*.

Fecal collections were made 32, 60, 84, and 108 hr. after the first treatment. (The results of each collection are given in Table II.)

Extraction of the Feces.—The feces of each collection were dried *in vacuo* at a temperature of 50°, whereafter the dried fecal output was homogenized in a Glencreston ball mill. (Afterward the drying process seemed to be unnecessary.) The homogeneous powder is suspended in 3 L. of 1 *N* hydrochloric acid and shaken for 1 hr. The suspension is filtered on a Büchner funnel, and the residue is treated again with 2 L. of distilled water. The filtrate, which is clear and deep-brown in color, is alkalized with 10 *N* sodium hydroxide (pH 10) and extracted with 4 + 3 + 3 L. of diethyl ether.

After concentration to about 1 L., the ether extract was extracted with 200 + 100 + 50 ml. of 0.1 *N* hydrochloric acid. The water layer was alkalized with 5 ml. of 10 *N* sodium hydroxide and extracted 3 times with 50 ml. of chloroform. The extract was concentrated by evaporation *in vacuo* to a volume of 20 ml., leaving a clear dark brown solution.

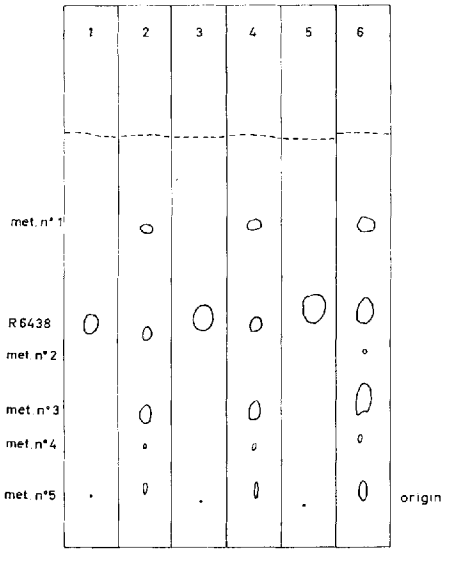


Fig. 1.—Thin-layer chromatogram of the concentrated fecal extract on Silica Gel G, 250 μ. Key: strips 1, 3, and 5: 1, 2, and 5 mcg. of R 6438; strips 2, 4, and 6: 1, 2, and 5 μl. of concentrated fecal extract.

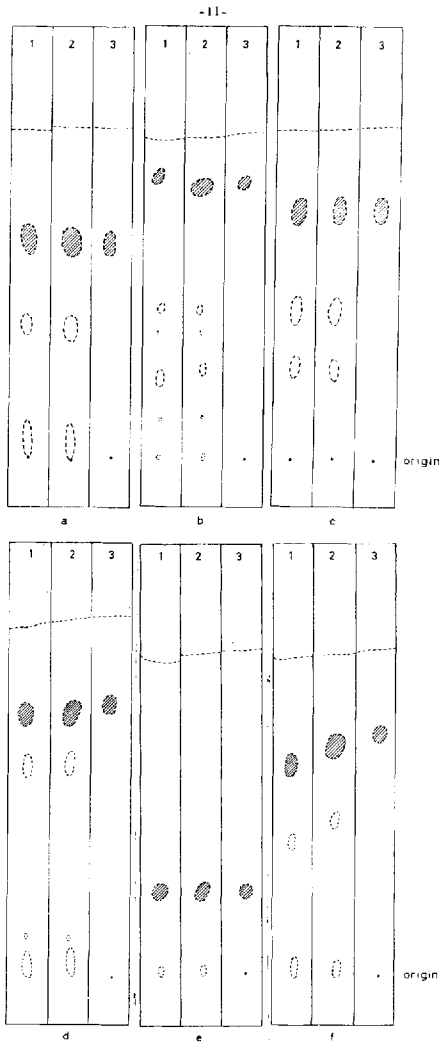


Fig. 2.—Identification of metabolite No. 1. Thin-layer chromatography of the concentrated extract on silica gel, 250 μ. Key: strip 1, 1 μl. of the concentrated extract; strip 2, a mixture of 1 and 3; strip 3, 1 mcg. of R 5987. Solvent systems are: a, methyl alcohol; b, chloroform–methyl alcohol (95:5 v/v); c, methyl alcohol–acetate buffer pH 4.7 (90:10 v/v); d, ethyl acetate; e, methylene chloride; f, methyl isobutyl ketone.

Thin-Layer Chromatography.—Small aliquots (1–5 μl.) of the concentrated extract were spotted on Silica Gel G chromatoplates (250 μ) and developed with a solvent system consisting of chloroform–methyl alcohol (95:5, v/v) (Fig. 1).

The air-dried chromatograms were sprayed with a modified Liebermann-Burchard reagent. This consists of ethyl alcohol–sulfuric acid, d. 1.84–acetic anhydride (80:10:10, v/v). Antazonite and its metabolites containing the thiophene ring system produce a blue color with this reagent after about 20 min. at a temperature of 110°.

At least six Liebermann-Burchard-active spots are found; two are very faint (metabolites No. 2 and 4).

Identification of Metabolites No. 1 and 5.—Metabolite No. 1 corresponds to 6-(2-thienyl)-imidazo[2,1-*b*]thiazole (R 5987) (Scheme I). This has been concluded after TLC on Silica Gel G with different moving liquids (Fig. 2): methyl alcohol, chloroform–methyl alcohol (95:5, v/v), methyl alcohol–acetate buffer pH 4.7 (90:10, v/v), ethyl acetate, methylene chloride, and methyl isobutyl ketone.

The R_f values of metabolite No. 1 correspond to those of the synthetically made R 5987.

Metabolite No. 5 was identified as deacetylated antazonite, 3-[2-hydroxy-2-(2-thienyl)-ethyl]-2-imino-thiazoline (R 6299). (See Scheme I.) The reference compound was chromatographed alone and co-chromatographed with the fecal extract. Three different moving liquids prove the structure of metabolite No. 5 (Fig. 3): methyl alcohol, ethyl acetate, and methyl alcohol–acetate buffer pH 4.7 (90:10, v/v).

Identification of Metabolite No. 3.—*Isolation Technique.*—This main metabolite was isolated by column chromatography on silica gel 0.05–0.20 mm. (E. Merck A. G., Darmstadt, 7734). An amount of 120 Gm. of the silica gel was suspended in methyl alcohol and poured in sections into a glass tube with an internal diameter of 3.0 cm. The column (35 cm. in length) was equilibrated at room temperature with about 200 ml. of methyl alcohol. The extract (about 15 ml.) was then placed on the column.

The chromatogram was first developed with methyl alcohol at a flow rate of 5 ml./hr., 10-ml. fractions being collected. After 1000 ml. of effluent, the developing solution was replaced by methyl alcohol containing 1% ammonia, of which 500 ml. was used.

Results of the Column Chromatography.—Each fraction of effluent was examined by thin-layer chromatography using 10-μl. quantities. The following separation was obtained: fractions 16 to 24, R 5987 and antazonite; fractions 23 to 64, the unknown metabolite (No. 3); fractions 106 to 124, deacetylated antazonite (R 6299).

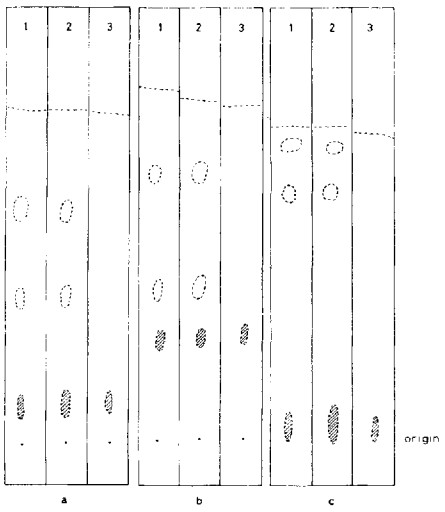


Fig. 3.—Identification of metabolite No. 5. Thin-layer chromatography of the concentrated extract on Silica Gel G, 250 μ. Key: strip 1, 1 μl. of the concentrated extract; strip 2, a mixture of 1 and 3; strip 3, 1 mcg. of R 6299. Solvent systems are: a, methyl alcohol; b, methyl alcohol–acetate buffer pH 4.7 (90:10 v/v); c, ethyl acetate.

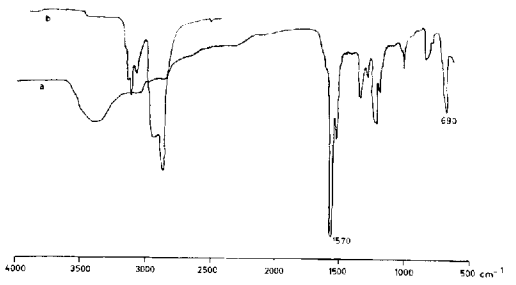


Fig. 4.—Infrared scan of one of the fractions 26–64 after evaporation. Key: a, KBr-disk; b, solution in carbon tetrachloride.

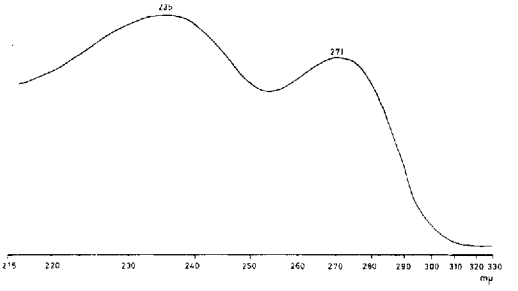
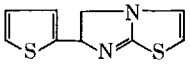


Fig. 5.—Ultraviolet scan of the same fraction (Fig. 4) in isopropyl alcohol.

Spectrophotometric Measurements.—U.V. and I.R. spectra of one of the fractions 26–64 were run. From the I.R. spectrum (Fig. 4) the following assignments could be made. The presence of —OH, =NH, and C=O is excluded. The 690 cm.$^{-1}$ band can be assigned to the γCH of the thiophene ring while the 1570 cm.$^{-1}$ band probably originates from a C=N group situated in a five-membered ring.

Examination of the U.V. spectrum (Fig. 5) revealed the presence of a nonconjugated thiophene ring (235 mμ). In this manner structure I



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could be deduced (5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole).

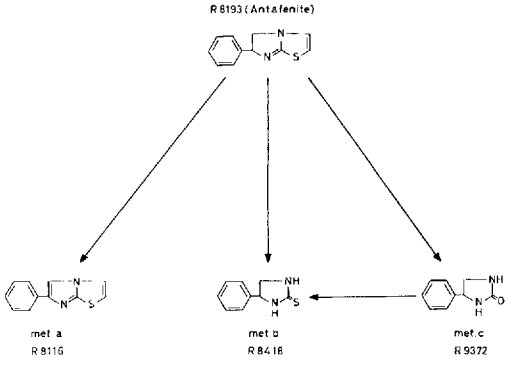
Crystallization and Analytical Results.—The fractions 26 to 60 were concentrated by evaporation *in vacuo*, and the remaining oily substance was crystallized as the oxalate salt from isopropyl alcohol.

The melting point after drying at 70° for 2 hr. was 191–192°.

Anal.—Calcd. for $C_9H_8N_2S_2.(COOH)_2$: C, 44.28; H, 3.38; N, 9.39; S, 21.50. Found: C, 44.05; H, 3.36; N, 9.55; S, 21.51.

The mixed melting point with the synthetically made oxalate (R 8025) was not depressed (192–193°). The ultraviolet spectrum (isopropyl alcohol) showed absorption bands at 237 mμ (ε 10,200) and 266 mμ (ε 9,320).

Possible Structure of Metabolites No. 2 and 4.—These two metabolites were not identified with certainty. Both metabolites could be degradation products of the major metabolite. This suggestion is supported by metabolic studies on 5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole (R 8193). The metabolic fate of R 8193 (antafenite) is represented in Scheme II.



Scheme II

Metabolites a and b were identified by thin-layer chromatography on Silica Gel G with several moving liquids. The spots were revealed with Dragendorff's reagent modified by Thies and Reuther (4).

Metabolite c was isolated by column chromatography on silica gel 0.05–0.20 mm. in the same manner as described above. The structure was elucidated by infrared spectrophotometry, and, after synthesis of the product, by thin-layer chromatography.

Comparison between the metabolic patterns of R 6438 (R 8141) and R 8193 suggests that analogous breakdown takes place, with the formation of analogous metabolites. The similarity between the R_f values of both groups of metabolites also confirms this suggestion (Fig. 6). It is concluded, therefore, that metabolite No. 2 could be 2-thio-5-(2-thienyl)-imidazolidine. Accordingly, metabolite No. 4 could be 2-oxo-5-(2-thienyl)-imidazolidine.

Anthelmintic Activity of R 8141 and R 6438.—One hen was treated with 100 mg. of the oxalate salt of metabolite No. 3 (R 8025). This corresponds to 70 mg. of the base. Examination of the feces showed the anthelmintic activity of the metabolite. In further parasitological studies the oxalate salt (R 8025) was replaced by the hydrochloride salt (R 8141).

The anthelmintic properties of the different substances in chickens can be summarized as follows.

(*a*) R 6438, 2-(acetyl-imino-3-[2-hydroxy-(2-thienyl)-ethyl]-thiazoline (antazonite). An oral dose of 160 mg./Kg. body weight has 100% effectiveness against *Heterakis* sp. and *Ascaridia* sp. Only 30% of *Capillaria* sp. are expelled.

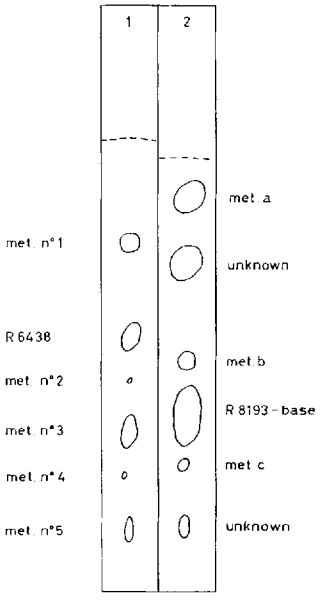


Fig. 6.—Comparative thin-layer chromatography of fecal extracts of chickens after oral treatment with R 6438, resp. R 8193. Key: strip 1, moving liquid–chloroform–methyl alcohol (95:5 v/v), spray reagent, Liebermann–Burchard; strip 2, moving liquid, chloroform–methyl alcohol (90:10 v/v); spray reagent, Dragendorff's reagent.

(*b*) R 8141, 5,6-dihydro-6-(2-thienyl)-imidazo-[2,1-*b*]thiazole (antienite). One oral dose of 40 mg./Kg. expels all *Heterakis* sp., *Ascaridia* sp., and *Capillaria* sp. from the chickens.

(*c*) R 8193, 5,6-dihydro-6-(2-phenyl)-imidazo-[2,1-*b*]thiazole (antafenite) has the same anthelmintic activity as R 8141, but the product is not so well tolerated.

(*d*) R 8299, 2,3,5,6-tetrahydro-6-phenyl-imidazo-[2,1-*b*]thiazole (tetramisole)[1] has been chosen for further parasitological and pharmacological work for several reasons. The dose required is less than 20 mg./Kg. body weight; the aqueous solution is sufficiently stable at room temperature; the product can be administered in several ways (orally, subcutaneously, or intramuscularly); and no side effects were observed (1).

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[1] Marketed under the trade names: Ripercol, Nilverm, and Citarin.