137. *Chemistry of Micrococcin* P. *Part I.*

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Micrococcin *P*, an antibiotic with therapeutic activity obtained from cultures of a spore-bearing bacillus of the *B. pumilus* group, gave on acid hydrolysis three groups of products, designated, for convenience, the " acid-insoluble fraction," the " ether-soluble fraction," and the " acid-soluble fraction." On treatment under esterification conditions the " acid-insoluble fraction " gave a dimethyl ester $C_{34}H_{33}O_5N_5S_4$ and a base (?)$C_{16}H_{19}O_3N_3S_3$, isolated as the picrate. The " ether-soluble fraction " consisted of a mixture of a substance $C_7H_7O_3NS$ and propionic acid, while the " acid-soluble fraction " contained ammonia, L-threonine, and a substance $C_8H_{12}O_2N_2S$, isolated as the hydrochloride. The substance $C_7H_7O_3NS$ has been identified by degradation and synthesis as 2-propionylthiazole-4-carboxylic acid (IV), and the substance $C_8H_{12}O_2N_2S$ as (+)-2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (XI) by conversion into (±)-2-(1-hydroxy-2-methylpropyl)-thiazole-4-carboxylic acid (X) and 2-*iso*butyrylthiazole-4-carboxylic acid (VIII), which have been synthesised. The molecular weight of micrococcin *P* has not been established satisfactorily by experiment, but estimates based on the amounts of hydrolytic fragments isolated place it in the region of 2200.

The probable biogenetic derivation of the compounds $C_7H_7O_3NS$ (IV) and $C_8H_{12}O_2N_2S$ (XI) from cysteine and α-aminobutyric acid, and cysteine and valine, respectively, is discussed.

An antibiotic with therapeutic activity was recently obtained [1] in this Institute from cultures of a spore-bearing bacillus of the *B. pumilus* group, isolated from soil collected in East Africa, and the present communication reports progress that has been made with the study of its chemistry. While this work was being carried out, our attention was drawn by Dr. E. P. Abraham to the similarity between the recorded properties [1] of the

[1] Fuller, *Nature*, 1955, **175**, 722.

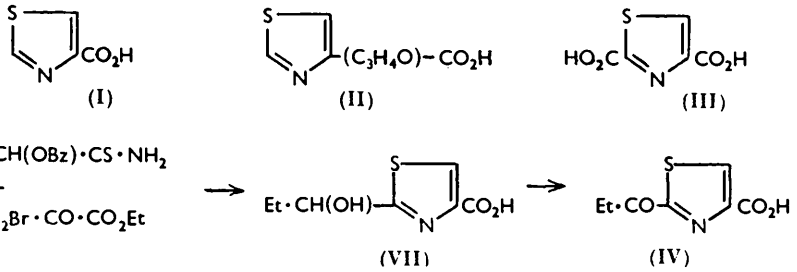
antibiotic produced by this species of *B. pumilus* and those [2] of an antibiotic produced by a species of *Micrococcus*, isolated from Oxford sewage, which had been named micrococcin [3] although nothing was known about its chemical nature.[2] Comparison of the properties and behaviour of these two substances, coming, as they do, from such widely divergent sources, has indeed demonstrated [4] a considerable degree of identity, or near-identity, between them. With substances of such a degree of complexity as these have proved to be, however, one cannot be confident in the present state of knowledge that they are identical in every detail, and it is possible that, as in other cases amongst antibiotics, we are dealing with two members of a group of closely related compounds. As the name micrococcin is already established in the literature, it may be undesirable to apply a specific trivial name to the antibiotic produced by the *B. pumilus* species in view of the relation between the two antibiotics that has emerged. On taxonomic grounds, however, the term micrococcin is too narrowly suggestive of source to be satisfactory as a name for the antibiotic produced by the *B. pumilus* species, but, in the circumstances, it may serve as a chemically generic name, and the *B. pumilus* antibiotic is therefore referred to [4] as micrococcin *P* in the absence of evidence to show that the two substances are completely identical.

On hydrolysis with hot 20% hydrochloric acid micrococcin *P* broke down to a series of products which were separated into three groups. One, the "acid-insoluble fraction," was separated at the end of the hydrolysis after dilution with water. The second, the "ether-soluble fraction," was isolated by continuous ether-extraction of the diluted aqueous acid mixture, and the third, the "acid-soluble fraction," was obtained on subsequent evaporation of the aqueous acid solution to dryness. These three fractions may now be considered in turn.

In contact with the aqueous acid mother-liquors the "acid-insoluble fraction" appeared to consist largely of a yellow crystalline substance, but washing with water discharged the yellow colour and left a buff, apparently amorphous, product, suggesting hydrolysis of a salt of a weak base. The "acid-insoluble fraction" amounted to about 40% of the weight of the original micrococcin *P*. It was insoluble in the usual organic solvents but dissolved readily in aqueous alkali. Treatment with boiling methanolic hydrogen chloride, or, better, with boiling methanol containing 2·5% of sulphuric acid effected solution slowly with subsequent separation of a crystalline solid. This proved to be a substance $C_{24}H_{23}O_5N_5S_4$ containing two methoxyl groups. Micrococcin *P* is methoxyl-free and the appearance of two methoxyl groups in the substance $C_{24}H_{23}O_5N_5S_4$ can be attributed to the esterification of two carboxyl groups. The yield, based on the "acid-insoluble fraction," amounted to 55%, or *ca.* 22·5% (as free acid) based on the original micrococcin *P*. The infrared absorption spectrum showed three strong bands in the carbonyl-stretching region, and these may be attributed to conjugated and unconjugated ester carbonyl groups ($\nu_{max.}$ 1710 and 1740 cm.$^{-1}$), and (?) an aryl (heterocyclic?) ketone or (?) hindered tertiary amide-carbonyl group ($\nu_{max.}$ 1690 cm.$^{-1}$); absorption bands at 1685—1690 cm.$^{-1}$ were subsequently observed in the infrared spectra of ketones containing the carbonyl group conjugated with the 2-position of the thiazole ring (see below), but tertiary amides do not usually absorb at quite such high frequency. The ultraviolet absorption spectrum was complex and obviously consisted of the sum of absorptions due to several isolated chromophores. The esterification mixture, after separation of the compound $C_{24}H_{23}O_5N_5S_4$, was found to contain a weakly basic substance, which separated on basification after concentration. It could not be crystallised but the ultraviolet absorption spectrum was similar to that of the compound $C_{24}H_{23}O_5N_5S_4$, and the infrared absorption spectra of the two also showed many similar features, except that that of the weak base had only two bands in the carbonyl-stretching region ($\nu_{max.}$ 1710 and 1730 cm.$^{-1}$). Analysis of a picrate gave figures suggesting the formula $C_{16}H_{19}O_3N_3S_3,C_6H_3O_7N_3$.

[2] Heatley and Doery, *Biochem. J.*, 1951, **50**, 247.
[3] Su, *Brit. J. Exp. Path.*, 1948, **29**, 473.
[4] Abraham, Heatley, Brookes, Fuller, and Walker, *Nature*, 1956, **178**, 44.

The " ether-soluble fraction " was apparently a mixture of two components, one of which, separated from the other by steam-distillation, was recognised by vapour-phase partition chromatography as propionic acid, and the other was a crystalline solid $C_7H_7O_3NS$, which could be sublimed in a vacuum. It accounted for *ca.* 8·3% of the original micrococcin *P*. Potentiometric titration indicated the presence of one acidic group of pK_a *ca.* 3·7, and the infrared absorption spectrum showed only one band (ν 1690 cm.$^{-1}$) in the carbonyl-stretching region suggestive of a conjugated carboxyl group. The ultraviolet absorption spectrum showed a single maximum at 282 mμ (log ε 3·69). At this stage cautious oxidation with alkaline potassium permanganate at room temperature followed by removal of manganese dioxide with sulphur dioxide gave a substance $C_4H_3O_2NS$, which agreed in m. p. with that recorded [5] for thiazole-4-carboxylic acid (I). Comparison with thiazole-4-carboxylic acid (I), prepared by the method of Erlenmeyer and Morel,[5] confirmed that the oxidation product was indeed that substance. The ultraviolet absorption spectrum of thiazole-4-carboxylic acid, however, showed a single maximum at 230 mμ (log ε 3·82), and it therefore appeared improbable that the substance $C_7H_7O_3NS$ could be a thiazole (II) with only one side-chain conjugated with the ring in view of the observed ultraviolet absorption at considerably longer wavelength. On treatment with 3N-sulphuric acid at 180° the substance $C_7H_7O_3NS$ gave propionic acid, identified by vapour-phase partition chromatography, and, again, thiazole-4-carboxylic acid (I). It gave a dinitrophenylhydrazone, however, and the infrared absorption spectrum of the methyl ester showed clearly the presence of two carbonyl groups, the absorption due to the carboxyl-carbonyl group having been shifted to higher frequency on esterification, away from the band attributable to a conjugated ketonic-carbonyl group, which had been obscured by the absorption due to the carboxyl-carbonyl group in the free acid. It was then found that, by avoiding the use of sulphur dioxide in the working up (see above), a second product was obtained, agreeing in its properties with an

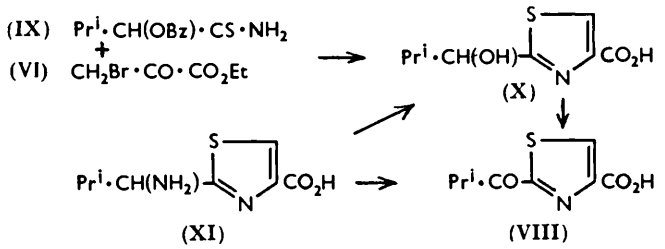


authentic specimen of thiazole-2 : 4-dicarboxylic acid (III), prepared by the method of Erlenmeyer, Junod, Guex, and Erne,[6] and the production of thiazole-4-carboxylic acid (I) in this oxidation as the main product is attributable to the extreme ease with which a carboxyl group can be lost from the 2-position in a thiazole-2-carboxylic acid.[6,7] It therefore appeared likely that the substance $C_7H_7O_3NS$ was 2-propionylthiazole-4-carboxylic acid (IV), and this was confirmed by comparison with an authentic specimen, synthesised by condensation of α-benzoyloxybutyrothioamide (V) with ethyl bromopyruvate (VI) followed by hydrolysis and oxidation of the resulting 2-1′-hydroxypropylthiazole-4-carboxylic acid (VII) to the keto-acid (IV) with sodium dichromate in acetic acid.

The " acid-soluble fraction " contained about 55% of the nitrogen originally present in the micrococcin *P*, and nitrogen in the form of ammonia in amount corresponding to

[5] Erlenmeyer and Morel, *Helv. Chim. Acta*, 1942, **25**, 1073.
[6] Erlenmeyer, Junod, Guex, and Erne, *ibid.*, 1948, **31**, 1345
[7] Kondo and Nagasawa, *J. Pharm. Soc. Japan*, 1937, **57**, 249; Erlenmeyer, Marbet, and Schenkel, *Helv. Chim. Acta*, 1945, **28**, 924; Schenkel, Marbet, and Erlenmeyer, *ibid.*, 1944, **27**, 1437; Schenkel and Schenkel-Rudin, *ibid.*, 1948, **31**, 924; Erne and Erlenmeyer, *ibid.*, p. 652; Boon, *J.*, 1945, 601.

about 23% of the total nitrogen present in the antibiotic. The presence of four substances reactive towards ninhydrin was revealed by paper chromatography, three of the spots being typical in colour and one atypical. Subsequent chromatography on the cation-exchange resin " Amberlite IR-120(H) " gave two pure components. The slowest-running substance in the paper chromatography was the one to leave the resin first in N-hydrochloric acid, and evaporation gave a lævorotatory hydrochloride, $C_4H_9O_3N$,HCl. This composition, the lævorotation, and the R_F value pointed to L-threonine or D-*allo*threonine hydrochloride. Authentic specimens of L-threonine and D-*allo*threonine were therefore converted into the hydrochlorides and comparison of the infrared absorption spectra showed conclusively that the substance obtained from micrococcin *P* was L-threonine, accounting for *ca.* 7·5% of the original antibiotic. The hydrochloride of L-threonine does not appear to have been described before but it is a highly crystalline substance and well suited for characterisation. The substance responsible for the atypical ninhydrin spot and one giving a closely associated and typical spot were removed from the column along with much ammonia. Separation of the ammonia, however, by a subsequent ion-exchange process left such a small amount of material as to suggest that the two substances in this fraction chromogenic towards ninhydrin may have been artefacts. Finally, elution with 3N-hydrochloric acid and evaporation of the effluent afforded a dextrorotatory hydrochloride, $C_8H_{12}O_2N_2S$,HCl, showing a maximum in its ultraviolet absorption spectrum at 234 mμ (log ε 3·81) [cf. thiazole-4-carboxylic acid (above), $\lambda_{max.}$ 230 mμ (log ε 3·82)]; it accounted (as free base) for *ca.* 14·4% of the original micrococcin *P*. On treatment in alkaline solution with dilute aqueous potassium permanganate under mild conditions the rate of oxidation slowed down considerably with the consumption of one atom of oxygen per molecule of $C_8H_{12}O_2N_2S$, to give a substance $C_8H_9O_3NS$, showing an ultraviolet maximum at 285 mμ (log ε 3·76). The ultraviolet and infrared absorption properties of this substance were very similar to those of the substance $C_7H_7O_3NS$ described above, and the analysis indicated the oxidation product to be a homologue of that substance. Furthermore, as



with the substance $C_7H_7O_3NS$, treatment of the compound $C_8H_9O_3NS$ with 3N-sulphuric acid at 180° gave thiazole-4-carboxylic acid (I), indicating the substance to be a 2-butyrylthiazole-4-carboxylic acid. The substance was in fact shown to be 2-*iso*butyrylthiazole-4-carboxylic acid (VIII), by comparison with an authentic specimen obtained by condensation of α-benzoyloxy*iso*valerothioamide (IX) with ethyl bromopyruvate (VI) followed by oxidation of the resulting 2-(1-hydroxy-2-methylpropyl)thiazole-4-carboxylic acid (X). Treatment of the substance $C_8H_{12}O_2N_2S$,HCl with nitrous acid gave an optically inactive product identical with synthetic (±)-2-(1-hydroxy-2-methylpropyl)thiazole-4-carboxylic acid (X). These observations show the substance $C_8H_{12}O_2N_2S$ to have been (+)-2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (XI). The conversion of (XI) into (VIII) by the action of alkaline potassium permanganate is attributable to dehydrogenation to the ketimine followed by its hydrolysis in analogy with the conversion,[8] in similar circumstances, of benzylanilines into benzylideneanilines and thence into aromatic aldehydes, and the racemisation observed in the nitrous acid reaction accords with

[8] D.R.P. 92,084; Friedländer, " Fortschritte der Teerfarbenfabrikation," 1899, **4**, 131.

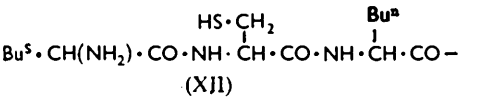
experience [9] of other deaminations in which an asymmetric centre involved is adjacent to an aromatic nucleus.

We have not yet established the molecular weight of micrococcin *P* satisfactorily by experiment, although Heatley and Doery [2] estimated the molecular weight of the original micrococcin by Barger's method " to be slightly greater than 2170 and definitely less than 2720," but they were operating " near the lower limit of workability of the method " as far as concentration was concerned. The amounts of the substances which we have isolated after hydrolysis of micrococcin *P* enable us to make a provisional estimate of the molecular weight as about 2200 as the following Table shows. The effect of isolating a hydrolysis product in less than 100% of the theoretical yield is reflected in that substance's appearing to account for a smaller proportion of micrococcin *P* than it should, and the molecular

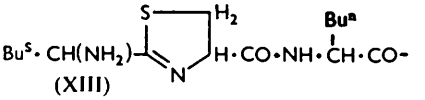
Fraction	Hydrolysis product	Apparent % of micrococcin *P* accounted for	*M* of hydrolysis product	Calc. *M* for micrococcin *P*	Mol. per mol. of micrococcin *P*
Acid-insoluble	Substance $C_{24}H_{22}O_5N_6S_4$ (calc. as free dibasic acid)	22·5	561	2500	1
	Substance $C_{16}H_{19}O_3N_3S_3$ (free base)	18·75	397	2120	1
Ether-soluble	Propionic acid	2·68	74	2750	1
	Substance $C_7H_7O_3NS$ (IV)	8·33	185	2200	1
Acid-soluble	L-Threonine	7·52	119	1580	2
	Substance $C_8H_{12}O_2N_2S$ (XI) (free base)	14·4	200	1390	2
	Ammonia	3·82	17	445	5

weight of the antibiotic calculated on the basis of the molecular weight of that particular hydrolysis product becomes artificially high. Inspection of the Table also shows that hydrolysis of micrococcin *P* probably produces one molecule of each of the first four hydrolysis products, two molecules each of L-threonine and the substance $C_8H_{12}O_2N_2S$ (XI), and five molecules of ammonia per molecule of antibiotic; it also suggests that about 10% of the antibiotic may yet have to be accounted for.

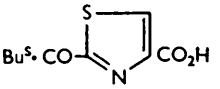
The isolation of 2-propionylthiazole-4-carboxylic acid (IV) and 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (XI) on hydrolysis of micrococcin *P* points to similar modes of biogenesis of these two fragments, which can be further illustrated by reference to the antibiotic bacitracin produced by certain strains of *Bacillus licheniformis*. Bacitracin *A*, a polypeptide containing one atom of sulphur per molecule, gives cysteine on complete hydrolysis [10] and yet shows no thiol reaction until, for example, after brief



(XII)



(XIII)



(XIV)

treatment with hot 0·5N-hydrochloric acid; [11] the same treatment also causes liberation of amide ammonia and loss of an ultraviolet absorption band at 254 mμ. The *N*-terminal amino-acid sequence of bacitracin *A* has been established [12] as *iso*leucylcysteinyl-leucyl-(XII), and the preceding observations have been rationalised [11,13] in terms of a thiazoline

[9] Brewster, Hiron, Hughes, Ingold, and Rao, *Nature*, 1950, **166**, 179; Ingold, " Structure and Mechanism in Organic Chemistry," G. Bell and Sons Ltd., London, 1953, p. 397.

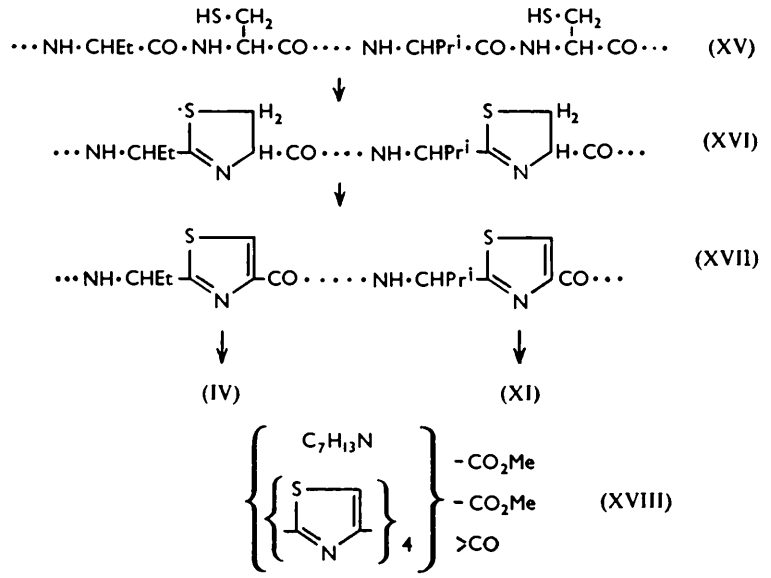
[10] Barry, Gregory, and Craig, *J. Biol. Chem.*, 1948, **175**, 485; Craig, Hausmann, and Weisiger, *ibid.*, 1952, **199**, 865.

[11] Newton and Abraham, *Biochem. J.*, 1953, **53**, 604.

[12] Lockhart and Abraham, *ibid.*, 1954, **58**, 633; Lockhart, Abraham, and Newton, *ibid.*, 1955, **61**, 534; Weisiger, Hausmann, and Craig, *J. Amer. Chem. Soc.*, 1955, **77**, 731.

[13] Cf. Craig, Hausmann, and Weisiger, *ibid.*, 1954, **76**, 2839; *J. Biol. Chem.*, 1953, **200**, 772; Porath, *Nature*, 1953, **172**, 871.

structure (XIII) for bacitracin *A*. Thiazoline structures have previously been postulated [14] as an explanation for masked thiol groups in proteins and there is spectral evidence for a thiazoline ring in glutathione under appropriate conditions.[15] The relevance of these observations to the biosynthesis of (IV) and (XI) is made still clearer by consideration of bacitracin *F*.[16] When bacitracin *A* is exposed in solution at pH 7 or slightly higher it is converted into bacitracin *F* with loss of nitrogen as ammonia and a shift in the maximum in the ultraviolet absorption spectrum from 254 to 290 mμ. On hydrolysis, the resulting bacitracin *F* gives no cysteine and, instead, the single sulphur atom emerges in a crystalline substance $C_9H_{11}O_3NS$,[17] which shows a maximum in its ultraviolet absorption spectrum at 285 mμ (log ε 3·76) and for which the structure (XIV) has been proposed.[17b] Although synthetic proof has not, as far as we are aware, been forthcoming, there can be little doubt



that this structure is correct, and the recorded light absorption and general properties [17] of the substance are in excellent agreement with those observed for 2-propionylthiazole-4-carboxylic acid (IV) and 2-*iso*butyrylthiazole-4-carboxylic acid (VIII), obtained by oxidation of (+)-2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (XI), derived from micrococcin *P*. The substances (IV), (VIII), and (XIV) are, in fact, adjacent members of a homologous series of 2-acylthiazole-4-carboxylic acids. The thiazole derivatives which we have isolated from micrococcin *P* are therefore considered to be derived from the incorporation of cysteine and the other requisite amino-acids into a peptide chain (XV) with subsequent conversion of cysteine and adjacent residues into thiazolines (XVI) and thence into thiazoles (XVII). In the case of 2-propionylthiazole-4-carboxylic acid (IV) the precursors are obviously cysteine and α-aminobutyric acid, and in the case of 2-(1-amino-2-methylpropyl)thiazole-4-carboxylic acid (XI) they are cysteine and valine. It is noteworthy that the *N*-terminal amino-acid sequence of bacitracin *A* on conversion into the end group of bacitracin *F* loses nitrogen as ammonia, to emerge ultimately on hydrolysis as the 2-acylthiazole-4-carboxylic acid (XIV), whereas with micrococcin *P* one thiazole is obtained as the keto-acid (IV) and the other as the undegraded 2-aminoalkylthiazole-4-carboxylic acid (XI). These fragments, (IV) and (XI), may be incorporated in micrococcin *P* in the form in which they are isolated after hydrolysis, or the appearance of one

[14] Linderstrøm-Lang and Jacobsen, *J. Biol. Chem.*, 1941, **137**, 443.
[15] Calvin, in "Glutathione. A Symposium," Academic Press Inc., New York, 1954, pp. 21—26.
[16] Craig, Weisiger, Hausmann, and Harfenist, *J. Biol. Chem.*, 1952, **199**, 259.
[17] (*a*) Hausmann, Weisiger, and Craig, *J. Amer. Chem. Soc.*, 1955, **77**, 730; (*b*) Weisiger, Hausmann, and Craig, *ibid.*, p. 3123.

as the ketonic and the other as the amino-derivative may be related to the position occupied in the molecule of the antibiotic and to the order of events during hydrolysis. Although our knowledge of the substance $C_{24}H_{23}O_5N_5S_4$ is as yet fragmentary the nitrogen : sulphur ratio, stability of the parent dibasic acid to further acid hydrolysis, light absorption properties, and other evidence are compatible with a polythiazole structure (XVIII) for this substance.

Experimental

M. p.s were observed on a microscope hot stage. Ultraviolet light absorption measurements were made in 95% ethanol. Infrared absorption spectra were determined in pressed potassium bromide, or potassium chloride, discs, except where otherwise stated.

Acid Hydrolysis of Micrococcin P.—Micrococcin *P* (5·4 g.) dissolved in 20% aqueous hydrochloric acid (150 c.c.) to give a bright yellow solution, which was heated under reflux for 16 hr. After about 1 hr. at the b. p. the solution suddenly became cloudy owing to the separation of a small quantity of a white crystalline solid, shown microscopically to consist of well-defined prisms. The amount of this precipitate increased during about 10 min. only, and then remained apparently unaltered throughout the remainder of the hydrolysis. After a further 2 hr. at the b. p. a further precipitation took place. This second precipitate consisted of bright yellow needles; it increased in amount during *ca.* 1 hr. and then remained constant. No other obvious changes took place during the hydrolysis. During the initial stage of the hydrolysis nitrogen was passed through the mixture and into aqueous barium hydroxide to detect any evolution of carbon dioxide, but none was apparent. No hydrogen sulphide was evolved during the hydrolysis.

The reaction mixture was finally poured into water (750 c.c.), and the crystalline yellow precipitate was collected. On washing with water the yellow colour was lost and the remaining buff-coloured solid, the " acid-insoluble fraction," was no longer obviously crystalline. The solid was then washed with acetone and ether, and dried (yield 2·2 g.).

The filtrate and washings were continuously extracted with ether for 16 hr. and the ether extract so obtained was dried and evaporated to give a product (0·62 g.), the " ether-soluble fraction," which was obviously a mixture of a crystalline solid and, from the odour and acidic reaction, a lower fatty acid.

The aqueous solution remaining after the ether-extraction was concentrated to dryness, redissolved in water, and again taken to dryness to give a deliquescent brown solid (2·8 g.), the " acid-soluble fraction."

Esterification of the " Acid-insoluble Fraction."—The " acid-insoluble fraction " (0·5 g.) was suspended in dry methanol (30 c.c.) containing concentrated sulphuric acid (1 c.c.) and the mixture was boiled under reflux for 8 hr. The solid slowly dissolved giving a clear solution, from which a crystalline solid subsequently separated on continued refluxing. The solution was cooled and the precipitate (0·29 g.) was collected and washed with methanol. The *substance* separated from chloroform–ether in colourless needles, m. p. 248—250° [Found : C, 49·3; H, 3·7; N, 11·7; S, 22·1; MeO, 10·3; *M* (Rast), 602, 613. $C_{24}H_{23}O_5N_5S_4$ requires C, 49·0; H, 3·7; N, 11·9; S, 21·8; 1MeO, 10·5%; *M*, 589]. Infrared light absorption : $\nu_{max.}$ 1740(s), 1710(s), 1690(s), 1585(m), 1560(m), 1515, 1490(m), 1465(m), 1445(s), 1415(m), 1375(m), 1345(m), 1325(m), 1305(m), 1280, 1245(s), 1215(s), 1190(s), 1115(m), 1095(s), 1060, 1045, 1020(m), 995(s), 985(m), 960(m), 930, 915, 905, 890(m), 855(m), 825, 800(m), 795(m), 775(m), 765(s), 745(s), 700, and 675 cm.$^{-1}$.

The filtrate and washings were concentrated to small bulk (*ca.* 10 c.c.), diluted with water (50 c.c.), and again concentrated (to 20 c.c.). This aqueous solution was made slightly alkaline with 2N-sodium hydroxide, and the precipitated brown solid (0·23 g.) was collected. A small quantity of this solid was made into a slurry with water, taken into solution by the addition of a few drops of dilute sulphuric acid, and treated with aqueous sodium picrate, affording a *picrate*, which separated from ethanol in yellow prisms, m. p. 155—159° (Found : C, 42·1; H, 3·5; N, 13·7; S, 15·2. $C_{16}H_{19}O_3N_3S_3,C_6H_3O_7N_3$ requires C, 42·2; H, 3·5; N, 13·4; S, 15·3%).

Examination of the " Ether-soluble Fraction."—The " ether-soluble fraction " (0·62 g.) was dissolved in water (25 c.c.) and steam-distilled until the distillate was only faintly acid. Aliquot parts (25 c.c.) of the total distillate (420 c.c.) were titrated against 0·1N-sodium hydroxide (Required : 1·15 c.c.; so total distillate contained 1·93 m-equiv. of acid). The distillate was

rendered slightly alkaline with aqueous sodium hydroxide, concentrated to small bulk (10 c.c.), acidified with dilute sulphuric acid, and extracted with ether. The dried ethereal solution was shown by vapour-phase partition chromatography to contain propionic acid, and the amount in the steam-distillate could then be estimated (1·93 m-equiv., 145 mg.).

The residue after steam-distillation was acidified slightly with dilute sulphuric acid and continuously extracted with ether. The ether solution, on drying and evaporation, afforded a buff-coloured solid (0·45 g.). Crystallisation from chloroform–light petroleum (b. p. 40—60°) gave small colourless needles which changed into regular prisms above *ca.* 140° and finally melted at 169—171° [Found : C, 45·5; H, 3·7; N, 7·3; S, 17·2; *C*-Me, 5·7; *M* (Rast), 192. $C_7H_7O_3NS$ requires C, 45·4; H, 3·8; N, 7·6; S, 17·3; 1*C*-Me, 8·1%; *M*, 185]. Light absorption, (*a*) ultraviolet : $\lambda_{max.}$ 282 mμ (log ε 3·69); (*b*) infrared : $\nu_{max.}$ 1690(s), 1475(m), 1405(m), 1380, 1360(m), 1340(m), 1280, 1255(m), 1230(m), 1100, 1090, 1010, 955(m), 900(m), 835, 800, 770(m), and 755(m) cm.$^{-1}$. Potentiometric titration (glass electrode) in aqueous solution against 0·02 N-sodium hydroxide showed the substance to have a pK_a of 3·7. The substance was subsequently shown by m. p., mixed m. p., and light absorption to be identical with the authentic *2-propionylthiazole-4-carboxylic acid* (IV) described below.

The *methyl ester*, obtained quantitatively by the action of diazomethane in ether, was a pale yellow crystalline solid; it sublimed at *ca.* 70° in a vacuum, affording colourless prisms, m. p. 87—90° (Found : C, 48·0; H, 4·8; N, 7·0. $C_8H_9O_3NS$ requires C, 48·2; H, 4·5; N, 7·0%). Light absorption, (*a*) ultraviolet : $\lambda_{max.}$ 282 mμ (log ε 3·70); (*b*) infrared : $\nu_{max.}$ 1725(s), 1685(s), 1550, 1505, 1485(m), 1460(m), 1435(m), 1410(m), 1380, 1360(m), 1340(m), 1220(s), 1105(m), 1085(m), 1010, 995(m), 930(m), 900(s), 840(m), 800, 775(s), and 735(m) cm.$^{-1}$; in chloroform solution the carbonyl-stretching bands were at 1730 and 1695 cm.$^{-1}$.

Oxidation of the Substance $C_7H_7O_3NS$ *with Alkaline Potassium Permanganate.*—The substance (44 mg.) was dissolved in 0·1N-sodium hydroxide (5 c.c.) and treated dropwise at room temperature with 2% aqueous potassium permanganate until a pink colour, stable for 5 min., was observed (Required : 3·8 c.c., *i.e.*, 75 mg. of potassium permanganate = 3 at. of oxygen per mol. of $C_7H_7O_3NS$). The oxidation mixture was divided into two parts, which were worked up separately as follows :

(i) To the one part, approximately half of the reaction mixture, aqueous sulphur dioxide solution was added drop by drop until the precipitated manganese dioxide dissolved. The clear colourless solution was rendered still more acidic by the addition of a few drops of dilute sulphuric acid and was then continuously extracted with ether. The dried extract gave on evaporation a white solid (11 mg.), and recrystallisation from chloroform–light petroleum gave colourless needles, m. p. 192—194° (Found : C, 37·4; H, 2·3; N, 10·3; S, 23·8. Calc. for $C_4H_3O_2NS$: C, 37·2; H, 2·3; N, 10·9; S, 24·8%). Light absorption, (*a*) ultraviolet : $\lambda_{max.}$ 230 mμ (log ε 3·82); (*b*) infrared : $\nu_{max.}$ 1675(s), 1490(m), 1445(s), 1400, 1330, 1280(s), 1215(s), 1110, 940(s), 890(m), 860(m), 830, 775, and 690(m) cm.$^{-1}$. The substance was shown by m. p., mixed m. p., and light absorption to be identical with an authentic specimen of thiazole-4-carboxylic acid.[5]

(ii) The remainder of the above oxidation mixture was freed from manganese dioxide by centrifugation. The clear solution was then acidified with dilute sulphuric acid and continuously extracted with ether for 3 hr. During this time the ethereal solution slowly deposited a white solid, which was collected and dried. In m. p. observations the not obviously crystalline solid changed at 140—144° into prisms, which subsequently melted at 190—192° (the m. p. of thiazole-4-carboxylic acid). Ultraviolet absorption : $\lambda_{max.}$ 265 mμ (log ε 3·57). The substance was shown by m. p. behaviour, mixed m. p., and infrared absorption spectrum to be identical with an authentic specimen of thiazole-2 : 4-dicarboxylic acid [6] (III). The ethereal filtrate afforded thiazole-4-carboxylic acid on evaporation.

Hydrolysis of the Substance $C_7H_7O_3NS$ *with* 3N-*Sulphuric Acid at* 180°.—The substance (50 mg.) was dissolved in 3N-sulphuric acid (5 c.c.) and heated in a sealed tube at 180° for 8 hr. After cooling, the solution was steam-distilled until the distillate was no longer acidic. The residual solution was continuously extracted with ether for 3 hr. and the dried extract gave on evaporation a colourless solid (25 mg.), which was identified as thiazole-4-carboxylic acid by m. p. and infrared absorption spectrum.

The steam-distillate was made slightly alkaline with aqueous sodium hydroxide solution, concentrated to small bulk (5 c.c.), acidified with dilute sulphuric acid, and continuously extracted with ether. On evaporation the dried extract afforded a small quantity of a liquid which was shown to be propionic acid by vapour-phase partition chromatography.

Synthesis of 2-Propionylthiazole-4-carboxylic Acid (IV).—(i) α-Benzoyloxybutyrothioamide [18] (V) (11·15 g.) was added slowly to ethyl bromopyruvate [5] (VI) (9·75 g.) cooled in ice. When all the solid had been added, the mixture was stirred and heated at 100° for 1 hr. Water was added to the cooled mixture and the whole was extracted three times with an equal volume of ether. Evaporation of the dried ethereal solution afforded a reddish-brown oil (16·0 g.), which could not be distilled in a vacuum without decomposition.

(ii) This oil (16·0 g.), crude ethyl 2-1′-benzoyloxypropylthiazole-4-carboxylate, was dissolved in alcohol (50 c.c.) and treated cautiously with an alcoholic solution (25 c.c.) of potassium hydroxide (5·6 g.). When all the alkali had been added the solution was boiled under reflux for 1 hr., and then taken to dryness on the water-bath in a vacuum. The residue was taken up in water and freed from an oily contaminant by extraction with ether. The clear aqueous solution was acidified with concentrated hydrochloric acid and extracted once with half its volume of ether. This ether extract, dried and evaporated, gave benzoic acid (3·5 g.) containing a small proportion of the required product. The aqueous phase was then continuously extracted with ether for 3 hr., and the dried extract, on evaporation, afforded a reddish-brown oil (6·1 g.).

(iii) The preceding product (6·1 g.), crude 2-1′-hydroxypropylthiazole-4-carboxylic acid (VII), was dissolved in glacial acetic acid (25 c.c.) (previously distilled from chromium trioxide) and treated with sodium dichromate (5 g.) in water (2·5 c.c.). The solution was heated at 100° for 2 hr. and then taken to dryness. Water was added and the solution was again taken to dryness, these operations being repeated twice more to ensure maximum removal of acetic acid. The solid remaining finally was taken up in water (25 c.c.) and extracted continuously with ether for 3 hr. The product (2·7 g.), isolated in the usual way, was crystallised from chloroform–light petroleum, affording small needles of 2-propionylthiazole-4-carboxylic acid (IV), which changed into regular prisms above *ca.* 140° and finally melted at 169—171° (Found: C, 45·0; H, 3·8; N, 7·4. Calc. for $C_7H_7O_3NS$: C, 45·4; H, 3·8; N, 7·6%).

Examination of the "Acid-soluble Fraction."—The deliquescent brown solid (2·8 g.), obtained as described above, was taken up in water (50 c.c.), part remaining undissolved. The insoluble material (0·11 g.) was separated and found to be readily soluble in alkali and in strong acid, but not in water or in organic solvents. It contained nitrogen and sulphur but no halogen, and a solution in weak alkali showed no significant ultraviolet light absorption. The aqueous solution was examined by paper chromatography (downward flow; 16 hr.) with the system, butan-1-ol–acetic acid–water (63 : 10 : 27). The paper was dried, sprayed with 0·1% ninhydrin solution in butan-1-ol, dried, and heated at 110° for 5 min., revealing the presence of four ninhydrin-reacting substances giving spots of R_F *ca.* 0·15 (violet), 0·20 (yellow) forming a cap to a spot of R_F 0·25 (violet), and 0·45 (violet). The spot of R_F 0·25 had a concave leading edge.

The aqueous solution was then applied to a column of "Amberlite IR-120(H)," which had been treated with 2N-sodium hydroxide and washed, and then treated with 2N-hydrochloric acid and washed with distilled water until the effluent was neutral. The aqueous solution was washed through the column with distilled water, and the effluent was collected. Evaporation left no residue, indicating complete retention of solutes on the column. The column was then eluted with N-hydrochloric acid and fractions (each 20 c.c.) were collected automatically. Alternate fractions were examined by paper chromatography as described above, samples (0·2 c.c.) being evaporated to dryness in a vacuum-desiccator on small convex Polythene discs, and the residues being applied to the paper in water (0·05 c.c.).

Fractions 1—8 contained no ninhydrin-reacting material and left no residue on evaporation.

Fractions 9—20 gave a violet spot of R_F 0·15.

Fractions 21—25 gave the violet spot of R_F 0·15, together with a second violet spot of R_F 0·25, which had, just above it and forming a cap to it, a yellow spot of R_F 0·20.

Fractions 26—47 gave the yellow spot and the second violet spot. There was no indication of any separation of the substances responsible for these spots; when one was strong so was the other.

Fractions 48—55 gave the same two spots, together with a trace of a third (violet), R_F 0·45.

Fractions 56—103 gave only the violet spot of R_F 0·45; as the material responsible for this spot was coming off the column slowly the strength of the eluting acid was increased to 3N.

[18] Olin and Johnson, *Rec. Trav. chim.*, 1931, **50**, 72.

Fractions 104—131 gave the violet spot of R_F 0·45, becoming progressively weaker.

The strength of the eluting acid was raised to 4N, but further fractions contained no ninhydrin-reacting material.

Fractions 9—20, on evaporation, afforded a crystalline solid (0·53 g.) which separated from methanol–ethyl acetate in long needles, m. p. 144—145°, $[\alpha]_D^{28}$ −11·8° (*c* 5·0 in H_2O) (Found: C, 30·8; H, 6·4; N, 8·8; Cl, 22·7. $C_4H_9O_3N$,HCl requires C, 30·9; H, 6·4; N, 9·0; Cl, 22·8%). The substance was shown to be L-*threonine hydrochloride* by comparison (m. p. and mixed m. p.) with an authentic specimen, prepared from the free amino-acid by evaporation with an excess of hydrochloric acid. The well-resolved infrared light absorption spectra of the two specimens were identical: $\nu_{max.}$ 1725—40(s), 1600(s), 1510(s), 1475, 1430(s), 1410(s), 1390(m), 1360, 1345, 1320, 1275(s), 1230(s), 1130(s), 1120(s), 1095(m), 1040(s), 995(m), 925(m), 875(m), 830(m), 805(m), 730 cm.$^{-1}$. The infrared light absorption spectrum of D-*allo*threonine hydrochloride, m. p. 152—153°, was markedly different and showed poorer resolution.

The hydrochloride isolated from the ion-exchange column was dissolved in ethanol and treated dropwise with pyridine. The precipitated amino-acid was washed with ethanol and had $[\alpha]_D^{22}$ −24·3° (*c* 3·0 in H_2O); West and Carter [19] record $[\alpha]_D^{26}$ −28·3° in water for L-threonine.

Fractions 26—47, on evaporation, gave a solid (0·73 g.) which consisted to a large extent of ammonium chloride. The solid was dissolved in water and applied to a column of "Amberlite IR-120(H)," chloride ions being rejected. Elution with 2N-ammonia solution and evaporation of the effluent then gave a small quantity of a brown liquid containing a few crystals and giving the yellow and violet spots with ninhydrin on paper chromatography.

Fractions 56—131, on evaporation, afforded crystalline 2-(1-*amino*-2-*methylpropyl*)*thiazole*-4-*carboxylic acid hydrochloride* (0·92 g.), which separated from methanol–ethyl acetate in colourless plates, which changed into small prisms above 200° and these melted at 264—267°, $[\alpha]_D^{23}$ +15·3° (*c* 0·69 in H_2O) (Found: C, 40·2; H, 5·6; N, 11·6; S, 13·2; Cl, 14·8. $C_8H_{13}O_2N_2S$,HCl requires C, 40·4; H, 5·5; N, 11·8; S, 13·5; Cl, 14·9%). Light absorption, (*a*) ultraviolet: $\lambda_{max.}$ 235 mμ (log ε 3·81); (*b*) infrared: $\nu_{max.}$ 1700(s), 1595(m), 1505(s), 1480(m), 1385(m), 1225(s), 1100, 1065, 995, 945, 870, 825(m), 760, 720, 700 cm.$^{-1}$.

In a separate experiment, micrococcin *P* (3·0 g.) was hydrolysed in the manner described above, and the "acid-soluble fraction" was isolated and taken up in water (150 c.c.) for the determination of total nitrogen and ammonia nitrogen. Aliquot parts (1 c.c.) were analysed for nitrogen by the normal micro-Kjeldahl procedure (Found: 5·28 c.c. of 0·02N-hydrochloric acid = 1·48 mg. of nitrogen), from which it followed from the nitrogen content of the antibiotic that the "acid-soluble fraction" contained *ca.* 55% of the total nitrogen present in the substance. Aliquot parts (2 c.c.) were basified and steam-distilled as in the Kjeldahl determination and the ammonia in the distillate was titrated (Found: 4·45 c.c. of 0·02N-hydrochloric acid = 1·24 mg. of nitrogen), from which it followed that volatile base (as ammonia) accounted for *ca.* 23% of the total nitrogen present in micrococcin *P*.

All the ninhydrin-reacting substances were found to be released within the first ½ hr. of hydrolysis.

Oxidation of the Substance $C_8H_{12}O_2N_2S$ *with Alkaline Potassium Permanganate.*—The hydrochloride $C_8H_{12}O_2N_2S$,HCl (100 mg.) was dissolved in 0·1N-sodium hydroxide (5 c.c.) and treated dropwise at room temperature with 2% aqueous potassium permanganate solution until a pink tinge, stable for 5 min., was observed (Required: 2·25 c.c., *i.e.*, 45 mg. of potassium permanganate = 1 at. of oxygen per mol. of $C_8H_{12}O_2N_2S$). The precipitated manganese dioxide was just dissolved by the passage of sulphur dioxide, and the clear solution so obtained was continuously extracted with ether for 2 hr. Evaporation of the dried extract afforded a crystalline solid (60 mg.) and crystallisation from chloroform–light petroleum gave colourless plates, m. p. 153—155° (Found: C, 48·0; H, 4·5; N, 7·3; S, 16·3. $C_8H_9O_3NS$ requires C, 48·2; H, 4·5; N, 7·1; S, 16·1%). Light absorption, (*a*) ultraviolet: $\lambda_{max.}$ 285 mμ (log ε 3·76); (*b*) infrared: $\nu_{max.}$ 1680(s), 1475(m), 1455(m), 1400(m), 1390(m), 1370, 1335(s), 1280, 1240(s), 1220(s), 1165(m), 1105(m), 960(s), 920(s), 885(m), 835(m), 770, 750(s) cm.$^{-1}$. The substance was subsequently shown by m. p., mixed m. p., and light absorption to be identical with the authentic 2-iso*butyrylthiazole*-4-*carboxylic acid* (VIII) described below.

The compound $C_8H_9O_3NS$ (45 mg.) was dissolved in 3N-sulphuric acid (5 c.c.) and the mixture was heated in a sealed tube at 180° for 8 hr. After cooling, the solution was steam-distilled until the distillate was no longer acidic, and continuous ether-extraction of the residual

[19] West and Carter, *J. Biol. Chem.*, 1937, **119**, 109.

solution afforded thiazole-4-carboxylic acid (*ca.* 10 mg.), identified by its m. p. and infrared absorption spectrum.

Action of Nitrous Acid on the Substance $C_8H_{12}O_2N_2S$,HCl.—A solution of the hydrochloride (120 mg.) in water (10 c.c.) containing concentrated hydrochloric acid (0·1 c.c.) was cooled in ice and treated with a solution of sodium nitrite (40 mg.) in water (1·5 c.c.). After 30 min. the mixture was continuously extracted with ether. The extract, dried and evaporated, gave a colourless solid (35 mg.), which separated from ether–light petroleum in prisms, m. p. 135—137°, $[\alpha]_D$ 0·0° (*c* 0·4 in H_2O). The infrared absorption spectrum and m. p. were identical with those of the authentic (±)-2-(1-hydroxy-2-methylpropyl)thiazole-4-carboxylic acid (X) described below.

Synthesis of 2-iso*Butyrylthiazole-4-carboxylic Acid* (VIII).—(i) *α-Benzoyloxyisovaleronitrile. iso*Butyraldehyde (24 g.), benzoyl chloride (47 g.), and powdered potassium cyanide (22 g.) were shaken with crushed ice (500 g.) for 1 hr., following the general procedure of Olin and Johnson;[18] a low-melting crystalline solid separated. The product was recovered in chloroform, washed with aqueous sodium hydrogen carbonate, dried, and fractionated, affording *α-benzoyloxyisovaleronitrile* (51 g., 75%), b. p. 113—116°/1 mm., n_D^{23} 1·5045 (Found: C, 70·7; H, 6·2; N, 6·8. $C_{12}H_{13}O_2N$ requires C, 71·0; H, 6·4; N, 6·9%). The substance crystallised at 0°.

(ii) *α-Benzoyloxyisovalerothioamide.* Hydrogen sulphide was bubbled for 24 hr. through a solution of the preceding nitrile (20·3 g.) in absolute alcohol (30 c.c.) containing triethanolamine (2 g.). The mixture was then poured into water (200 c.c.), a crystalline solid separating. After standing overnight the precipitate was collected, and recrystallisation from aqueous methanol afforded *α-benzoyloxyisovalerothioamide* as fine needles or plates (23·5 g.), m. p. 105—106° (Found: C, 60·7; H, 6·6; N, 5·7. $C_{12}H_{15}O_2NS$ requires C, 60·8; H, 6·3; N, 5·9%).

(iii) *2-(1-Hydroxy-2-methylpropyl)thiazole-4-carboxylic acid.* α-Benzoyloxy*iso*valerothioamide (2·37 g.) was condensed in the manner described above for the lower homologue with ethyl bromopyruvate (1·95 g.), and the resulting crude ethyl 2-(1-benzoyloxy-2-methylpropyl)-thiazole-4-carboxylate was a reddish-brown oil (3·1 g.). It was dissolved in ethanol (10 c.c.) and treated with ethanolic potassium hydroxide (1·0 g. in 5 c.c.). The solution was kept overnight and then evaporated to dryness on the steam-bath. The residue was taken up in water and freed from a small quantity of insoluble oil by extraction with ether. The aqueous phase was then acidified and extracted once with an equal volume of ether. The extract, dried and evaporated, gave an oil which solidified when scratched. It was extracted six times with boiling light petroleum to remove benzoic acid, and the ultimate residue was crystallised from ether–light petroleum, affording (±)-2-(1-*hydroxy*-2-*methylpropyl*)*thiazole*-4-*carboxylic acid* (X) as prisms (0·45 g.), m. p. 134—135°, subsequently raised to 136—138°. The remaining aqueous solution was continuously extracted with ether for 2 hr., affording a further quantity (0·54 g.) of the same substance (Found: C, 47·8; H, 5·8; N, 6·7. $C_8H_{11}O_3NS$ requires C, 47·8; H, 5·5; N, 7·0%). Light absorption, (*a*) ultraviolet: $\lambda_{max.}$ 235 mμ (log ε 3·80); (*b*) infrared: $\nu_{max.}$ 1705(s), 1495(m), 1390(m), 1375(m), 1220(s), 1100(m), 1045(m), 1015(m), 950, 865, 810, and 740(m) cm.$^{-1}$. The compound was identical with the product obtained by the action of nitrous acid on the substance $C_8H_{12}O_2N_2S$,HCl (above).

(iv) The preceding substance (0·5 g.) was oxidised in acetic acid with sodium dichromate in the manner described for the lower homologue (above), affording, on isolation, an oil (0·22 g.), which readily crystallised. Recrystallisation from chloroform–light petroleum gave 2-iso*butyrylthiazole-4-carboxylic acid* as plates, m. p. 153—154° (Found: C, 48·4; H, 4·5; N, 6·7. $C_8H_9O_3NS$ requires C, 48·2; H, 4·5; N, 7·0%).

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