The Bacterial Pigment from *Pseudomonas lemonnieri*.[1] Part 2. The Synthesis of 3-n-Octanamidopyridine-2,5,6-trione: the Structure and Synthesis of Lemonnierin

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Oxidation of 3-n-octanamido-5-aminocitrazinic acid (8; R = NH_2) with iron(III) chloride gave a mixture of 3-n-octanamidopyridine-2,5,6-trione (1; R = H) and lemonnierin (22; $R^1 = R^2$ = H) identical with the blue pigment from our strain of *Ps. lemonnieri*. Various derivatives of 3-n-octanamidocitrazinic acid and of (1; R = H) are described.

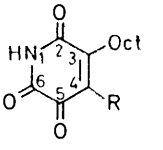
Condensation of (1; R = H) with the citrazinic acid (8; R = NH_2) gave 3-(6-hydroxy-5-octanamido-2-oxo-1,2-dihydro-3-pyridylimino)-6-hydroxy-5-octanamido-2-pyridone (14) (*cf.* Knackmussl[3,4]) which differs from lemonnierin but may be converted into lemonnierin, with which it is isomeric, by copper(II) acetate–pyridine. Lemonnierin thus appears, at least in part, to be a di-free radical, of type (22; $R^1 = R^2$ = H), with g = 2.004. Experiments with deuteriated intermediates together with the synthesis of the corresponding acetyl analogue (17) of the imine (14) and the acetyl analogue (25) of lemonnierin, substantiate the structure (22; $R^1 = R^2$ = H) for our pigment.

Imines of type (14) are converted into the corresponding 3,3'-bipyridylidenequinone (21) by hot acetic acid, thus providing a new and improved route to these quinones.

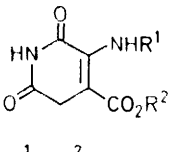
In the preceding paper,[1] we reported the structure (1; R = H) of a major degradation product of the blue bacterial pigment, lemonnierin, produced by *Ps. lemonnieri*. Additionally, a formal, total synthesis of (1; R = H) was described.[1] The present paper describes an improved synthesis of (1; R = H) together with the synthesis of lemonnierin; since this synthesis did not initially define the structure of the pigment the second part of this paper is concerned with this aspect of our problem.

Acylation of 3-aminocitrazinic acid[2] (3-amino-2,6-dioxo-1,2,3,6-tetrahydropyridine-5-carboxylic acid (2) with n-octanoyl chloride gave 3-n-octanamidocitrazinic acid. The n.m.r. spectrum (see Experimental section) exhibited a signal (in CF_3CO_2H) at τ 2.70 (s, H) corresponding to the C-5 proton of structure (5). Since this signal was absent from the spectrum in CF_3CO_2D the equilibrium (3) ⇌ (5) must obtain in acidic solution. In accord with this conclusion treatment of 3-n-octanamidocitrazinic acid with diazomethane gave methyl 3-n-octanamido-2,6-dimethoxycitrazinate (6), whilst reaction with benzaldehyde gave the derivative (7). A variety of oxidising agents failed to produce, from (7), any detectable quantity of the trione (1; R = H). Treatment of 3-n-octanamidocitrazinic acid with nitrous acid gave the 5-nitroso-derivative (8; R = NO). Attempts to hydrolyse this with acid gave the 4-n-octanamido-5-hydroxy-isoxazolopyridine-3,7-dione (10): collateral evidence for this structure was provided by the conversion of (8; R = NO), presumably in the tautomeric form (9) into the isoxazolopyridine (10), by *NN'*-dicyclohexylcarbodi-imide.

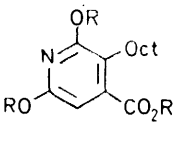
Attempts to reduce 5-nitrosocitrazinic acid (8; R = NO) to the corresponding 5-amino-derivative (8; R = NH_2) using a wide selection of reagents failed: however, with ammonium polysulphide we obtained the unstable 5-amino-acid (8; R = NH_2). In attempts to convert (8; R = NH_2) into the trione (1; R = H), (8; R = NH_2) was treated with nitrous acid; the product was a stable crystalline, solid which is formulated as the diazo-derivative (8; R = N_2), or perhaps more appropriately as the corresponding zwitterion. Efforts to hydrolyse this to the trione gave intractable



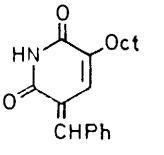
(1)



(2) $R^1 = R^2$ = H
(3) R^1 = CO[CH$_2$]$_6$Me, R^2 = H
(4) R^1 = COMe, R^2 = H



(5) R = H
(6) R = Me



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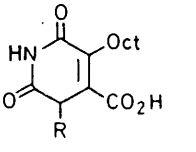

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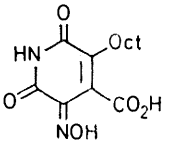

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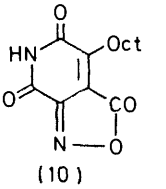

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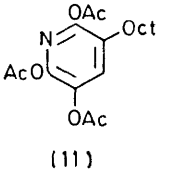


(11)

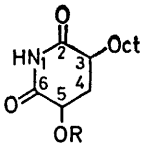
Oct = NH·CO[CH$_2$]$_6$Me

mixtures, whilst treatment of (8; R = NH_2) with numerous oxidising agents furnished a consistent succession of failures. Ultimately, a solution of 3-n-octanamido-5-aminocitrazinic acid (8; R = NH_2) in sodium hydroxide solution was oxidised (under nitrogen) with

aqueous ferric chloride solution to yield (*a*) the trione (1; R = H) in 10% yield, and (*b*) a blue solid (see later).

With the trione (1; R = H) readily available, it was further investigated. Thus, reductive acetylation with zinc–acetate anhydride gave 3-n-octanamido-2,5,6-triacetoxypyridine (11), whilst catalytic hydrogenation gave 3-n-octanamido-5-hydroxypiperidine-2,6-dione (12; R = H). This last compound readily regenerated the parent trione (1; R = H) on oxidation with chromic oxide. From general principles together with the n.m.r. spectra of (12; R = H) and its 5-*O*-acetate (12; R = Ac) the relative configuration and conformation were assigned. Thus, in (12; R = H) the coupling constants of 11.5 and 7.0 Hz for the C-5 proton suggest axial–axial (11.5 Hz) and axial–equatorial (7.0 Hz) coupling for this proton which must thus be axial and hence the C-5-hydroxy equatorial. Since, on general principles the C-3-n-octanamido-residue will also be equatorial, (12; R = Ac) may be represented as in (13). The n.m.r. spectrum of the 5-*O*-acetate (12; R = Ac) provided collateral support for this conclusion.

Purification of the blue fraction (b), from the oxidation of the 5-aminocitrazinic acid (8; R = NH_2) with ferric chloride gave a product, *mirabile dictu*, identical with natural lemonnierin,[1] although the method of synthesis did not immediately and completely define its structure. Soon after our synthesis, Knackmuss and co-workers [3,4] assigned the structure (14) to a blue pigment isolated



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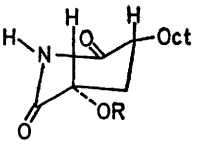

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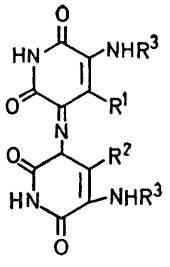

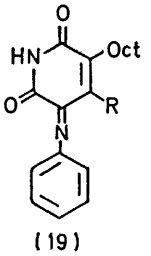

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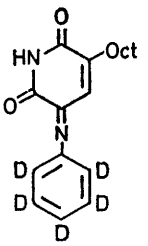


(20)

(14) $R^1 = R^2 = H, R^3 = CO[CH_2]_6Me$

(15) $R^1 = D, R^2 = H, R^3 = CO[CH_2]_6Me$

(16) $R^1 = H, R^2 = D, R^3 = CO[CH_2]_6Me$

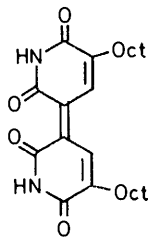
(17) $R^1 = R^2 = H, R^3 = COMe$

(18) $R^1 = R^2 = D, R^3 = CO[CH_2]_6Me$

Oct = $NH{\cdot}CO[CH_2]_6Me$

from a strain of *Ps. lemonnieri*. Because of numerous points of non-congruence between our conclusions and those of the German authors, including the crystallinity of (14) [3,4] (which we have confirmed) and the amorphous nature of our lemonnierin but more especially the absence from the n.m.r. spectrum of our pigment of a signal corresponding to a vinylic proton, the following extensions of our work were developed.

Thus the trione (1; R = H) was condensed with a variety of primary aromatic amines to yield derivatives of type (19; R = H), none of which was intensely



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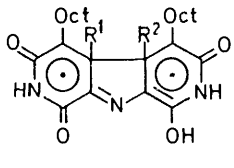
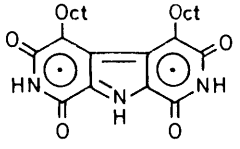


(22)

Oct = $NH{\cdot}CO[CH_2]_6Me$



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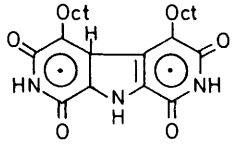

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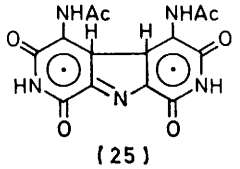

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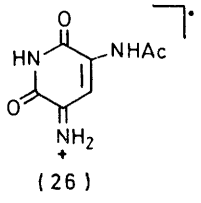

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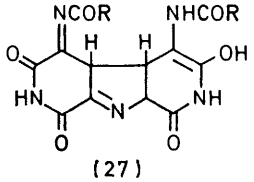

(27)

coloured and all of which clearly exhibited a signal in the n.m.r. spectrum in the region τ 1.7—2.1 as required by the presence of a vinylic proton. Any remaining dubiety on this point was removed by the synthesis of the derivative (20) by condensation of (1; R = H) with heptadeuterioaniline. This derivative exhibited the requisite vinylic proton signal at τ 2.10. Condensation of the trione (1; R = H) with the 5-aminocitrazinic acid (8; R = NH_2), in methanol containing a trace of hydrochloric acid gave (14) which also exhibited the requisite vinylic proton signal in the n.m.r. spectrum, whilst condensation of (1; R = D) with (8; R = NH_2) gave the monodeuterio-imine (15) which is tautomeric with (16). Similar tautomerism obtains with all imines of this type (see later).

To leave no doubt concerning the validity of our n.m.r. assignments 3-n-octanamido-5-aminocitrazinic acid (8; R = NH_2) was oxidised using deuteriated reagents to yield (*a*) 4-deuterio-3-n-octanamidopyridine-2,5,6-trione (1; R = D), which exhibited no vinylic proton signal and (*b*) dideuteriolemonnierin (22; $R^1 = R^2 = D$) (see later).

Condensation of (1; R = D) with aniline gave the derivative (19; R = D), devoid of a vinylic proton signal in the n.m.r. spectrum.

Oxidation of (15) with chromic oxide gave a mixture of the trione (1; R = H) and the 4-deuterio-derivative (1; R = D) in the ratio of 11 : 9 (*m/e* 266 : *m/e* 267), thereby establishing that the trione moiety is derived approximately equally from both ends of the substrate, and hence the tautomerism, (15) ⇌ (16). Knackmuss [3,4] determined the n.m.r. spectrum of the imine (14) in dimethyl sulphoxide at 80 °C; we found this not possible; (14) rapidly decomposed under these conditions. The preparation [3] of the imine (14) by refluxing a mixture of the trione (1; R = H) and 3-n-octanamido-5-aminocitrazinic acid in acetic acid gave, in addition to (14), substantial quantities of the bipyridylidenequinone (21) (contrast Knackmuss [3]). This result is not surprising since we have observed that the imine (14) may be converted, almost quantitatively, into the bipyridylidenequinone (21), in boiling acetic acid: *mutatis mutandis* the *N*-acetylimine (17) behaved similarly. This process provides a more convenient route than those previously available [5] to these bipyridylidenequinones.

The amine (14) exhibited u.v. and i.r. spectra indistinguishable from those of our lemonnierin: the n.m.r. spectra were, however, different as already indicated. Although Knackmuss [4] failed to obtain a parent molecular ion from (14), we experienced no such difficulty; oxidation of (14) as for lemonnierin gave the same trione (1; R = H). In agreement with Knackmuss,[4] attempts to methylate (14) gave only intractable material (contrast the successful methylation of lemonnierin [1]).

It thus appeared reasonably certain that the two pigments although similar were not identical and that despite certain dubieties the pigment reported by Knackmuss is probably represented by the structure (14), and that our lemonnierin may have a structure of type (22; $R^1 = R^2 = H$) together with possible, equivalent zwitterionic structures. This conclusion involved *inter alia* (*a*) the possible conversion of (14) into (22; $R^1 = R^2 = H$) by a ' coupling ' reaction and (*b*) that lemonnierin should be a di-radical. The e.s.r. spectrum of lemonnierin, determined on the solid, gave a broad intense peak (100 G), with $g = 2.004$, and confirmed our hypothesis, which we now sought to establish by the conversion of (14) into (22; $R^1 = R^2 = H$). Initially, a variety of conditions, including (i) treatment of a solution of (14) in acetic acid with oxygen at 20 °C during 15 h, (ii) treatment of a solution of (14) in acetic acid with iron(III) chloride as in the preparation of (1; R = H) which partially converted (14) into the bipyridylidenequinone (21), (iii) the action of 1M-hydrochloric acid at 100 °C during 45 min, or 0.25M sulphuric acid at 60 °C during 2 h, which gave unchanged imine (14) and quinone (21), (iv) boiling a solution of (14) in pyridine for 1½ h, which gave unchanged material, and (v) refluxing a solution of (14) in pyridine–sodium acetate for 1½ h which caused no change; all failed to achieve our objective. However, when (14) was refluxed for 1 h in boiling pyridine containing a trace of copper(II) acetate conversion into (22; $R^1 = R^2 = H$) occurred in high yield (*cf.* ref. 6). The product from this reaction was identical (including n.m.r.) with natural lemonnierin and with the product obtained from the 5-aminocitrazinic acid (8; $R = NH_2$) by oxidation with iron(III) chloride. E.s.r. measurements indicate that (14) also exhibits some (di)-free radical character ($g = 2.004$), an observation compatible with its ready conversion into (22; $R^1 = R^2 = H$).

Collateral chemical evidence for the difference between (14) and our lemonnierin resides in that (*a*) whilst (14) was converted in high yield in boiling acetic acid into the bipyridylidenequinone (21), lemonnierin was stable under these conditions and was recovered unchanged, and (*b*) hydrogenation of lemonnierin gave only intractable products [contrast the behaviour of imines of type (14)] which furnish [4] the corresponding bipyridylidenequinone.

Thus lemonnierin could be represented as (22; $R^1 = R^2 = H$), (23), or (24). A distinction in favour of (22; $R^1 = R^2 = H$) and of possible equivalent zwitterionic structures was established by several mutually supportive methods. Thus, oxidation of lemonnierin with chromic oxide using deuteriated reagents gave the trione (1; R = H), in which no trace of the 4-deuterio-derivative (1; R = D) could be detected using high-resolution mass spectrometry: hence (23) would appear to be excluded. A distinction between (22; $R^1 = R^2 = H$) and (24) was achieved as follows.

Condensation of 3-n-octanamidocitrazinic acid with the 4-deuteriotrione (1; R = D) in methanol, and subsequent decarboxylation of the product gave the monodeuterio-imine (15), having τ 0.83 (s, C=C-*H*, 1 H) and *m/e* 516 ($C_{26}H_{36}DN_5O_6$) and *m/e* 515 ($C_{26}H_{37}N_5O_6$) in the ratio of 1 : 4, thus indicating an 80% incorporation of deuterium. Cyclisation of (15) with copper(II) acetate gave the corresponding monodeuterio-lemonnierin (22; $R^1 = H$, $R^2 = D$): the mass spectrum, with *m/e* 515 ($C_{26}H_{37}N_5O_6$) and *m/e* 516 ($C_{27}H_{36}DN_5O_6$) in the ratio of 3 : 7, indicated a deuterium content of 70% and hence the retention of most of the deuterium from the parent imine (15). Oxidation of (22; $R^1 = H$, $R^2 = D$) gave the trione (1; R = H) and the 4-deuterio-trione (1; R = D) in a ratio of 9 : 11. This also shows that the trione moiety is derived from each ' end ' of lemonnierin as for the imine (*q.v.*).

Similarly, condensation of 4-deuterio-3-n-octanamidopyridine-2,5,6-trione (1; R = D) with 3-n-octanamidocitrazinic acid (2) in methan[^{2}H]ol in the presence of deuterium chloride, and decarboxylation of the resultant product gave the imine (18). The mass spectrum of this showed *m/e* 515 ($C_{26}H_{37}N_5O_6$), 516 ($C_{26}H_{36}DN_5O_6$), and *m/e* 517 ($C_{26}H_{35}D_2N_5O_6$) in the ratio of 1 : 3 : 6. Cyclisation with copper(II) acetate of this isotopically labelled mixture gave deuteriated lemonnierin having *m/e* 515, 516, and 517 in the ratio of 1 : 1 : 2, *i.e.* 50% of the lemonnierin contained two deuterium atoms [as in

(22; $R^1 = R^2 = D$)] as opposed to 60% in the precursor; hence it may be concluded that the label is essentially retained during cyclisation.

This result is satisfyingly close to the composition of the deuteriated lemonnierin obtained from the iron(III) chloride–deuterium oxide–deuterium chloride oxidation of 3-n-octanamido-5-aminocitrazinic acid previously described. The *m/e* 515, 516, and 517 ratio for this preparation of pigment was 1 : 1 : 2.

To provide additional evidence for the structure (22; $R^1 = R^2 = H$) the corresponding acetyl analogue (17) (*cf.* ref. 3) was synthesised by two methods. 3-Acetamido-5-aminocitrazinic acid [prepared from 3-acetamidocitrazinic acid (4)] was warmed in acetic acid to yield the imine (17) which was coupled, using copper(II) acetate, to yield (25). In the second synthesis, the 3-acetamido-5-aminocitrazinic acid was oxidised with iron(III) chloride to yield the same pigment (25), together with 3-acetamidopyridine-2,5,6-trione. The mass spectrum of (25) (at 325 °C) showed the parent ion, *m/e* 347.086 8 ($C_{14}H_{13}N_5O_6$ requires 347.086 6), and [*m/e* −42] and [*m/e* −84] corresponding to the loss of one and two acetyl residues respectively. The e.s.r. spectrum ($g = 2.004$) confirmed the free-radical nature of (25). The n.m.r. spectrum was devoid of vinylic proton signals, but exhibited (in CF_3CO_2D), a non-replaceable signal at τ 7.48 (s, 2 H): the analogous signals in lemonnierin would be obscured by the methylene envelope signals of the octanoyl residues. Collateral evidence for the difference between (17) and (25) [and hence for the relationship of (14) and (25; $R^1 = R^2 = H$)] was provided by the mass spectrum of (17), which in addition to the mass ion peak at *m/e* 347 ($C_{14}H_{13}N_5O_6$) (low intensity) showed, in contrast to the spectrum of (25), additional intense peaks at *m/e* 182 ($C_7H_8N_3O_3$) [corresponding to (26)], 111, 139, and 154. These observations are completely compatible with structure (25), and in agreement with the deuterium-labelling experiments described for lemonnierin.

Hence it is clear that our specimen of lemonnierin differs from (14) [3,4] and probably has structure (22; $R^1 = R^2 = H$), together with contributions from equivalent zwitterionic structures. Structures of type (27) appear to be excluded by the n.m.r. evidence, particularly that of the acetyl derivative (25). It is additionally clear that whilst lemonnierin may occur naturally as a salt with various inorganic cations (*cf.* ref. 1), these inorganic ions do not constitute a necessary, integral component of the structure of the pigment.

One point remains: is our pigment an artefact arising from the extraction process which involved boiling pyridine in the presence almost certainly of trace metals, including copper? An unequivocal answer is not possible: but the circumstantial evidence, namely our inability to extract our pigment from the undried, ruptured bacterium with solvents (contrast Knackmuss [3,4]) indicates fairly convincingly that (22; $R^1 = R^2 = H$) is not an artefact

EXPERIMENTAL

The e.s.r. spectra were measured using a Decca X3 Spectrometer with a 100-kHz field modulation and a Newport 11-in magnet, type M4X, at 20—25 °C. N.m.r. spectra were determined at 60 MHz, using the solvents indicated. Mass spectra were obtained using an AEI 902 mass spectrometer with a resolving power of 10 000 and valley definition of 10%.

3-n-Octanamidocitrazinic Acid and Derivatives.—3-Aminocitrazinic acid (3.4 g) dissolved in sodium hydroxide solution (20 ml, 16%) was shaken in nitrogen for 1 h, with the addition of n-octanoyl chloride (3.5 g). Additional sodium hydroxide solution (8 ml) and acid chloride (6 g) were added during a further hour. Two hours later the solution was adjusted to pH 7 with acetic acid and the solution filtered: the filtrate was acidified by the addition of concentrated hydrochloric acid. The resultant, off-white, microcrystalline precipitate was collected, dried, and exhaustively extracted with ether to yield *3-n-octanamidocitrazinic acid* (4.5 g), m.p. 250 °C (decomp.) (Found: C, 56.6; H, 6.9; N, 9.3. $C_{14}H_{20}N_2O_5$ requires C, 56.7; H, 6.8; N, 9.5); τ (in CF_3CO_2H) 9.1 (t, *J* 5 Hz, CH_2–CH_3, 3 H), 8.6 (m, 5 × CH_2, 10 H), 7.2 (m, $COCH_2$, 2 H), and 2.7 (s, vinyl proton, H). This compound was sparingly soluble in the usual organic solvents and could not be readily recrystallised.

Methyl 3-n-octanamido-2,6-dimethoxycitrazinate, prepared by the action of ethereal diazomethane on a suspension of the parent acid in ether during 24 h, at 0 °C, formed prisms, m.p. 85 °C from ether–light petroleum (b.p. 40—60 °C) (Found: C, 60.5; H, 7.7; N, 8.4. $C_{17}H_{26}N_2O_5$ requires C, 60.3; H, 7.7; N, 8.3%); τ (in $CDCl_3$) 9.1 (t, *J* 5 Hz, CH_2CH_3, 3 H), 8.66 (m, 5 × CH_2, 10 H), 7.6 (t, *J* 7 Hz, $COCH_2$, 2 H), 6.12 (s, OCH_2, 3 H), 6.08 (s, OCH_3, 3 H), 6.0 (s, OCH_3, 3 H), 3.32 (s, Ar-*H*, H), and 1.0 (s, N*H*, H). The signal at τ 1.0 slowly disappeared on deuteriation.

A mixture of 3-n-octanamidocitrazinic acid (1.5 g), pyridine (20 ml), and benzaldehyde (1 ml) was kept at 20 °C during 15 h, when the crystalline precipitate was collected and purified from benzene to yield *3-n-octanamido-5-benzylidenecitrazinic acid* (1.7 g) as yellow needles, m.p. 195—196 °C (Found: C, 65.5; H, 6.2; N, 7.2. $C_{21}H_{24}N_2O_5$ requires C, 65.6; H, 6.3; N, 7.3%); τ (in $CDCl_3$) 9.12 (t, 5 Hz, CH_2CH_3, 3 H), 8.7 (m, 5 × CH_2, 10 H), 7.5 (t, *J* 7 Hz, $COCH_2$, 2 H), 3.6 (s, Ar C*H*=C, 1 H), 2.66 (m, Ar-*H*, 5 H), 0.8 (s, N*H*CO, H), and −2.3 (CO–N*H*–CO, and CO_2H). The signals at τ 0.8 and −2.3 slowly collapsed on addition of D_2O.

3-n-Octanamido-5-nitrosocitrazinic Acid.—A solution of 3-n-octanamidocitrazinic acid (5.9 g) in aqueous sodium hydroxide (32 ml; 5%) was treated with a solution of sodium nitrite (32 ml; 5%) at 5 °C. Dropwise addition during 45 min, of hydrochloric acid (3M; 25 ml) gave *3-n-octanoyl-5-nitrosocitrazinic acid* (5.5 g) as pale green prisms, m.p. 245—250 °C (decomp.) (Found: C, 51.4; H, 5.8; N, 12.8. $C_{14}H_{19}N_3O_6$ requires C, 51.7; H, 5.9; N, 12.9%); τ (in CF_3CO_2H) 9.08 (t, *J* 5 Hz, CH_2CH_3, 3 H), 8.6 (m, 5 × CH_2, 10 H), and 7.1 (t, *J* 5 Hz, $COCH_2$, 2 H); $\lambda_{max.}$ (ethanol) 265 nm (log ε 3.98).

A solution of this nitroso-derivative (0.65 g) in acetone (25 ml) containing concentrated hydrochloric acid (0.1 ml) was kept at 20 °C during 48 h. After removal of the solvent at 20 °C the residue was purified by t.l.c. on silica from ethanol–chloroform (1 : 19) to yield *4-n-octanamido-5-hydroxyisoxazolo*[3,4-c]*pyridine-3,7-dione* (0.1 g) as prisms, m.p. 197 °C (Found: C, 54.4; H, 5.5; N, 13.4. $C_{14}H_{17}$-

N_3O_5 requires C, 54.7; H, 5.6; N, 13.7%); τ ($CDCl_3$) 9.1 (t, *J* 5 Hz, CH_2CH_3, 3 H), 8.6 (m, 5 × CH_2, 10 H), and 7.1 (t, *J* 7 Hz, $COCH_2$, 2 H).

The same derivative (0.23 g) was produced when a solution of 3-n-octanamido-5-nitrosocitrazinic acid (0.32 g) in dioxan (8 ml) was treated with a solution of *N,N'*-dicyclohexylcarbodi-imide (0.23 g) in dioxan (3 ml). After 24 h, at 20 °C the reaction mixture was filtered and the filtrate evaporated to yield the isoxazole, identical with the previous preparation.

3-n-Octanamido-5-aminocitrazinic Acid.—A solution of 3-n-octanamido-5-nitrosocitrazinic acid (3.25 g) in aqueous 1M-sodium hydroxide (25 ml) was treated with 10% ammonium polysulphide solution (12 ml) during 2 h, at 20 °C. The mixture was then clarified (under nitrogen) and the filtrate acidified with 3M-hydrochloric acid (20 ml) to yield a grey precipitate. This was washed, dried, and extracted with carbon disulphide (to remove sulphur) to yield *3-n-octanamido-5-amiocitrazinic acid* (2.2 g) as almost colourless microcrystals, m.p. >300 °C (Found: C, 53.7; H, 6.9; N, 13.3. $C_{14}H_{21}N_3O_5$ requires C, 54.0; H, 6.8; N, 13.5%). Knackmuss *et al.*[4] discuss the use of this substance but record no method of preparation and no physical constants.

A solution of this amino-acid (1.56 g) in 0.5M-sodium hydroxide (14 ml) was treated at 0 °C (under nitrogen) with a solution of 5% sodium nitrite (14 ml), followed by the addition of 3M-hydrochloric acid (15 ml), to give (as a micro-crystalline precipitate) *3-n-octanamido-5-diazocitrazinic acid* (1.0 g), m.p. >300 °C (Found: C, 52.4; H, 5.7; N, 16.9. $C_{14}H_{18}N_4O_5$ requires C, 52.2; H, 5.6; N, 17.4%).

3-n-Octanamido-5-hydroxypiperidine-2,6-dione.—A suspension of 3-n-octanamidopyridine-2,5,6-trione (0.26 g) in ethyl acetate (20 ml) containing 5% palladium–charcoal (0.2 g) was shaken in an atmosphere of hydrogen when the yellow solution rapidly became colourless. Purified from methanol, *3-n-octanamido-5-hydroxypiperidine-2,6-dione* (0.19 g) formed small needles, m.p. 165 °C (Found: C, 58.0; H, 8.3; N, 10.4%; M^+ 270. $C_{13}H_{22}N_2O_4$ requires C, 57.8; H, 8.2; N, 10.4%; *M* 270); τ ($CDCl_3$) 9.1 (t, *J* 5 Hz, CH_6CH_3, 3 H), 8.6 (m, 5 × CH_2, 10 H), 7.4 (t, *J* 7 Hz, $COCH_2$, 2 H), 7.1 (m, CH·CH_2·CH, 2 H), 4.85 (m, CH_2CH·NH, 1 H), and 3.9 (dd, *J* 11.5 Hz and *J* 7.0 Hz, CH–OH, 1 H).

The corresponding *5-acetate* separated from ethanol as prisms, m.p. 153 °C (Found: C, 58.0; H, 7.8; N, 8.9. $C_{15}H_{24}N_2O_5$ requires C, 57.7; H, 7.7; N, 9.0%); τ ($CDCl_3$) 7.85 (s, $COCH_3$, 3 H) and 4.35 (dd, *J* 12 Hz and *J* 6 Hz, CH–OAc, 1 H).

Oxidation of 3-n-octanamido-5-hydroxypiperidine-2,6-dione with (*a*) chromic oxide in acetone or (*b*) chromium trioxide–pyridine, regenerated the parent pyridine-2,5,6-trione in high yield.

3-n-Octanamido-2,5,6-triacetoxypyridine.—A mixture of the trione (1; R = H) (0.13 g), acetic anhydride (8 ml), and zinc dust (0.7 g) was heated at 80 °C during 1½ h. Isolated in the normal manner, *3-n-octanamido-2,5,6-triacetoxypyridine* (0.17 g) formed prisms, m.p. 141 °C from chloroform–light petroleum (b.p. 60—80 °C) (Found: C, 57.8; H, 6.6; N, 6.9. $C_{19}H_{26}N_2O_7$ requires C, 57.9; H, 6.6; N, 7.1%); τ ($CDCl_3$) 9.1 (t, *J* 5 Hz, CH_2CH_3, 3 H), 8.7 (m, 5 × CH_2, 10 H), 7.75 (m, $COCH_2$, and 3 × $OCCH_3$, 11 H), 2.6 (s, Ar-*H*, 1 H), and 1.3 (s, NH·CO, 1 H, exchangeable + D_2O).

Oxidation of 3-n-Octanamido-5-aminocitrazinic Acid with Ferric Chloride.—A solution of ferric chloride (1.3 g) in 5M-hydrochloric acid (7 ml) was added to a stirred solution of 3-n-octanamidocitrazinic acid (3.11 g) in 1M-sodium hydroxide solution (25 ml) at 20 °C (under nitrogen). After 4 h, the reaction temperature was raised to 35—40 °C and the reaction allowed to proceed for an additional 20 h. The cooled, dark blue mixture was extracted with ether (7 × 30 ml) and the combined extracts washed with water. The ethereal extract rapidly deposited an insoluble, blue substance (0.6 g) which was washed with acetone and found to be indistinguishable from natural lemonnierin (Found: C, 60.4; H, 7.1; N, 13.3%; M^+ 515.275 0. $C_{26}H_{37}N_5O_6$ requires C, 60.6; H, 7.2; N, 13.6%; *M*, 515.274 8). The u.v., i.r., and n.m.r. spectra were identical with the spectra of the natural pigment. Oxidation with Jones' reagent gave the 2,5,6-trione (1; R = H).

The filtrate remaining after removal of the lemonnierin was dried and evaporated to give a brown residue which was chromatographed from benzene on silica. Elution with ethyl acetate–benzene (1 : 19) gave 3-n-octanamido-pyridine-2,5,6-trione (0.32 g) identical with the product obtained by oxidation of lemonnierin (Found: C, 58.7; H, 6.9; N, 10.4. Calc. for $C_{13}H_{18}N_2O_4$: C, 58.6; H, 6.8; N, 10.5%). Continuing elution of the column furnished n-octanamide (0.05 g), m.p. 105 °C identical with an authentic specimen (Found: C, 66.9; H, 12.0; N, 9.9. Calc. for $C_8H_{17}NO$: C, 67.1; H, 12.0; N, 9.8%).

Repetition of this oxidation using 3-n-octanamido-5-aminocitrazinic acid (1.55 g) in deuterium oxide (>99% purity) (15 ml), containing dissolved sodium (0.03 g) and anhydrous ferric chloride (0.65 g) dissolved in deuterium oxide (5 ml) and deuterium chloride (38%; 2 ml) gave (*a*) *dideuteriolemonnierin* (0.3 g) (Found: C, 60.6; H, 7.2; N, 13.2. $C_{26}H_{35}D_2N_5O_6$ requires C, 60.4; H, 7.2; N, 13.5%): the mass spectrum exhibited peaks at *m/e* 515, 516, and 517 in the ratio of 1 : 1 : 2 corresponding to $C_{26}H_{37}N_5O_6$, $C_{26}H_{36}DN_5O_6$, and $C_{26}H_{35}D_2N_5O_6$, and (*b*) *4-deuterio-3-n-octanamidopyridine-2,5,6-trione* formed yellow prisms, m.p. 161 °C (Found: C, 58.3; H, 6.6; N, 10.6. $C_{13}H_{17}DN_2O_4$ requires C, 58.4; H, 6.8; N, 10.5%): τ ($CDCl_3$) 9.1 (t, *J* 5 Hz, CH_2CH_3, 3 H), 8.66 (m, 5 × CH_2, 10 H), 7.45 (t, *J* 7 Hz, $COCH_2$, 2 H), 1.6 (s, NHCO, H), and 0.95 (s, CONH·CO, H). The signals at τ 1.6 and 0.95 slowly collapsed on addition of D_2O. The mass spectrum exhibited peaks at *m/e* 267 and 266 in the ratio of 4 : 1, corresponding to $C_{13}H_{17}DN_2O_4$ and $C_{13}H_{18}N_2O_4$, respectively.

Oxidation of Lemonnierin with Non-protonating Reagents.—Synthetic lemonnierin (0.1 g) suspended in deuterium oxide (2 ml) was oxidised with a solution prepared from chromic oxide (5.2 g), deuterium oxide (15 ml), and dideuteriosulphuric acid (2.3 ml). The resultant oxidation mixture was diluted with deuterium oxide (20 ml) and then extracted with methylene chloride. A specimen of natural pigment was similarly oxidised. In each case the resultant 3-n-octanamidopyridine-2,5,6-trione was identical with an authentic specimen: the mass spectrum of each specimen showed the absence of deuterium.

3-n-Octanamido-5-phenyliminopyridine-2,5,6-trione.— A mixture of the trione (1; R = H) (0.05 g), xylene (6 ml), and aniline (0.02 g) was refluxed for 2½ h, and then evaporated to dryness *in vacuo*. Purified from methylene chloride–light petroleum (b.p. 60—80 °C) the *5-phenylimine* (0.06 g) formed orange prisms, m.p. 176 °C (Found: C, 66.7; H, 6.9; N, 12.4%; M^+ 341. $C_{19}H_{23}N_3O_3$ requires C, 66.8; H, 6.8; N, 12.3%; *M* 341); τ ($CDCl_3$) *inter alia*, 3.1—2.6 (m, Ar-*H*, 5 H), 2.15 (s, =C–*H*, 1H), 1.85 (s, NH·CO, 1H, replaceable + D_2O), and 1.43 (s, OC·NH·CO, 1H, replaceable + D_2O);

$\lambda_{max.}$ (ethanol) 260 (log ε 4.25), 350 (3.95), and 430 nm (3.80).

The same product (0.03 g) was obtained when (1; R = H) (0.05 g), methanol (4 ml), aniline (0.02 g), and methanolic hydrochloric acid (0.1 ml) were kept for 40 h, at 20 °C.

Prepared similarly from (1; R = H) but using [$^2H_7$]-aniline the 5-[$^2H_2$]*phenylimine* formed orange prisms, m.p. 176 °C (Found: C, 65.6; H, 6.5; N, 12.0%; M^+ 346. $C_{19}H_{18}D_5N_3O_3$ requires C, 65.9; H, 6.7; N, 12.1%; *M* 346). The n.m.r. spectrum was devoid of the signals at τ 3.1—2.6 but retained a signal at τ 2.1 (s, =C–*H*, 1 H).

The 5-(*p-tolylimine*) formed orange prisms, m.p. 188 °C from methylene chloride–light petroleum (b.p. 60—80 °C) (Found: C, 67.4; H, 7.0; N, 11.7. $C_{20}H_{25}N_3O_3$ requires C, 67.6; H, 7.1; N, 11.8%): the n.m.r. spectrum ($CDCl_3$) showed signals, *inter alia*, at τ 7.65 (m, $COCH_2$, C_6H_4–CH_3, 5 H), 3.15—2.70 (m, Ar-*H*, 4 H), and 2.1 (s, =C–*H*, 1 H).

The 5-(*p*-NN-*dimethylaminophenylimine*) formed purple prisms, m.p. 202 °C from methylene chloride–light petroleum (b.p. 60—80 °C) (Found: C, 65.6; H, 7.2; N, 14.6. $C_{21}H_{28}N_4O_3$ requires C, 65.6; H, 7.3; N, 14.6%). The n.m.r. spectrum exhibited signals, *inter alia*, at τ 6.95 [s, $-N(CH_3)_2$, 6 H], 3.3—2.7 (m, Ar-*H*, 4 H), and 1.75 (s, =C–*H*, 1 H); $\lambda_{max.}$ (ethanol) 260 (log ε 4.42), 350 (3.95), and 580 nm (4.35).

The 5-(*phenylhydrazone*) of (1; R = H) formed orange needles, m.p. 200—201 °C from methylene chloride (Found: C, 64.0; H, 6.7; N, 15.5 $C_{19}H_{24}N_4O_3$ requires C, 64.0; H, 6.8; N, 15.7%).

4-*Deuterio*-3-*n-octanamido*-5-*phenyliminopyridine*-2,5,6-*trione* (19; R = D).—Prepared from 4-deuterio-3-n-octanamido-2,5-dihydropyridine-2,5,6-trione (*q.v.*) (0.1 g), aniline (0.04 g), in boiling xylene (12 ml) during 2½ h, this 4-*deuterio*-5-*phenylimine* (0.12 g) formed orange prisms, m.p. 176 °C (Found: C, 66.4; H, 6.7; N, 12.4. $C_{19}H_{22}DN_2O_3$ requires C, 66.7; H, 6.8; N, 12.3%): the n.m.r. spectrum was devoid of the vinylic proton signal at τ 1.75.

3-(6-*Hydroxy*-5-*n-octanamido*-2-*oxo*-1,2-*dihydro*-3-*pyridylimino*)-6-*hydroxy*-5-*n-octanamido*-2-*pyridone* (14).—(*a*) A suspension of the trione (1; R = H) (0.27 g) and 3-n-octanamido-5-aminocitrazinic acid (0.31 g) in methanol (25 ml) containing 0.05% of hydrogen chloride was kept at 20 °C during 40 h. The resultant crystalline, blue precipitate was collected, washed with methanol, dried, and heated at 80 °C *in vacuo* for 2 h to yield the crystalline title compound (0.36 g) as blue-bronze plates, m.p. *ca.* 300 °C (decomp.) after purification by extraction (in the dark) with methylene chloride (Found: C, 60.1; H, 7.1; N, 13.3. Calc. for $C_{26}H_{37}N_5O_6$: C, 60.6; H, 7.2; N, 13.6%). Knackmuss [3] records m.p. *ca.* 288 °C. The amine exhibited signals in the n.m.r. spectrum ($CDCl_3$) at τ 9.06 (t, 5 Hz, 2 × CH_2-CH_3, 6 H), 8.6 (m, 10 × CH_2, 20 H), 7.25 (t, *J* 7 Hz, 2 × $COCH_2$, 4 H), and 0.85 (s, 2 × =C–*H*, 2 H); $\lambda_{max.}$ 640 nm (log ε 4.75) in pyridine or acetic acid. The mass spectrum exhibited peaks at *m/e* 515, 389, and 263 corresponding to $C_{26}H_{37}N_5O_6$, $C_{18}H_{23}N_5O_5$, and $C_{10}H_9N_5O_4$.

(*b*) Repetition of the literature process [3] for the preparation of this amine gave (14) and 6,6′-dihydroxy-5,5′-di-n-octanamido-3,3′-bipyridylidene-2,2′-quinone (21) in approximately equal proportions.

(*c*) A solution of 3-n-octanamido-5-aminocitrazinic acid (0.16 g) in acetic acid (5 ml) was refluxed for 15 min. The solid product which crystallised on cooling was a mixture (0.11 g) of (14) and (21) in approximately equal proportions.

(*d*) A solution of compound (14) (0.05 g) in acetic acid (3 ml) was refluxed for 6 h. Next day the crystalline precipitate was separated and identified (i.r., u.v., n.m.r., and mass spectra) as the quinone (21) (0.04 g).

(*e*) A solution of 3-n-octanamido-5-aminocitrazinic acid (0.1 g) in acetic acid (4 ml) was refluxed for 7 h. Next day the crystalline precipitate (0.8 g) was identified (i.r., u.v., n.m.r., and mass spectrum) as the diquinone (21).

Oxidation of the 4-*Pyridone* (14).—Finely divided amine (0.15 g) was oxidised by the addition (with grinding) of Jones' reagent. After the discharge of colour the product was isolated by extraction with ether (after dilution with water) to yield the 2,5,6-trione (1; R = H) (0.12 g), identical (m.p., mixed m.p., and i.r.) with an authentic specimen.

Conversion of the Amine (14) *into Lemonnierin*.—A solution of the amine (14) (0.085 g) in pyridine (8 ml) containing cupric acetate (0.008 g) was refluxed for 1¼ h, cooled, and diluted with water (10 ml). The resultant blue, non-crystallisable solid (0.065 g) was identical (i.r., u.v., n.m.r., and mass spectrum) with lemonnierin.

3-(6-*Hydroxy*-5-*n-octanamido*-2-*oxo*-1,2-*dihydro*-3-*pyridylimino*)-4-*deuterio*-6-*hydroxy*-5-*n-octanamido*-2-*pyridone* (15).—A mixture of 4-deuterio-3-octanamido-pyridine-2,5,6-trione (0.265 g) and 3-n-octanamido-5-aminocitrazinic acid (0.3 g) in methanol (25 ml) containing 0.05% hydrogen chloride was maintained at 20 °C for 40 h. The crystalline, blue precipitate of the title *compound* (0.36 g) had an n.m.r. spectrum exhibiting signals at τ 9.1 (t, *J* 5 Hz 2 × CH_2CH_3, 6 H), 8.6 (m, 10 × CH_2, 20 H), 7.25 (t, *J* 7 Hz, 2 × $OC{\cdot}CH_2$, 4 H), and 0.83 (s, =C*H*, 2 H). The mass spectrum exhibited peaks at *m/e* 516 and 575 corresponding to $C_{26}H_{36}DN_5O_6$ and $C_{26}H_{37}N_5O_6$ in the ratio of 4 : 1 (Found: C, 60.0; H, 7.1; N, 13.3. $C_{26}H_{36}DN_5O_6$ requires C, 60.4; H, 7.2; N, 13.6%).

Oxidation of this amine (0.2 g) with chromic oxide as for lemonnierin gave the trione (1; R = H) 0.045 g), m.p. and mixed m.p. 160 °C. The mass spectrum showed peaks at *m/e* 267 ($C_{13}H_{17}DN_2O_4$) and 266 ($C_{13}H_{18}N_2O_4$) in the ratio of 9 : 11.

Cyclisation of this mono-deuteriated amine (0.085 g) in pyridine (8 ml) containing cupric acetate (0.008 g) gave a mixture (0.065 g) of lemonnierin and monodeuterio-lemonnierin (22; R^1 = H, R^2 = D). The mass spectrum showed peaks at *m/e* 516 ($C_{26}H_{36}DN_5O_6$) and 515 ($C_{26}H_{37}N_5O_6$) in the ratio of 3 : 7.

Oxidation of this mixture (0.2 g) of lemonnierin and monodeuteriolemonnierin (22; R^1 = H, R^2 = D) gave a mixture of the trione (1; R = H) and the 4-deuterio-trione (1; R = D). The mass spectrum showed relative abundance peaks at *m/e* 267 ($C_{13}H_{17}DN_2O_4$) and 266 ($C_{13}H_{18}N_2O_4$) in the ratio of 9 : 11.

3-(4-*Deuterio*-6-*hydroxy*-5-*n-octanamido*-2-*oxo*-1,2-*dihydro*-3-*pyridylimino*)-4-*deuterio*-6-*hydroxy*-5-*n-octanamido*-2-*pyridone* (18).—A solution of 4-deuterio-3-n-octanamido-pyridine-2,5,6-trione (0.27 g) and 3-n-octanamido-5-aminocitrazinic acid (0.31 g) in methan[^{2}H]ol (25 ml) containing a trace of deuterium chloride was kept at 20 °C for 40 h, when the crystalline, blue precipitate (0.36 g) was collected, washed with methan[^{2}H]ol and dried to yield the title *amine* (Found: C, 60.1; H, 7.2; N, 131. $C_{26}H_{35}D_2N_5O_6$ requires C, 60.4; H, 7.2; N, 13.5%); the i.r. and u.v. spectra were almost indistinguishable from the spectra of undeuteriated material. The n.m.r. spectrum ($CDCl_3$) exhibited signals at τ 9.05 (t, *J* 5 Hz, CH_2CH_3, 3 H), 7.6 (m, 2 × O*H*, 10 × CH_2, 24 H), and 7.25 (t, *J* 7 Hz, 2 × $COCH_2$,

4 H). The ratio of *m/e* 515, 516, and 517 was 1 : 3 : 6—corresponding to $C_{26}H_{37}N_5O_6$, $C_{26}H_{36}DN_5O_6$, and $C_{26}H_{35}D_2N_5O_6$.

Cyclisation of this dideuterioamine (0.075 g) in boiling pyridine (6 ml) containing cupric acetate (0.007 g) during 1½ h gave *dideuteriolemonnierin* (22; $R^1 = R^2 = D$) (0.06 g). The mass spectrum displayed peaks at *m/e* 515, 516, and 517, in the ratio of 1 : 1 : 3 corresponding to $C_{26}H_{37}N_5O_6$, $C_{26}H_{36}DN_5O_6$, and $C_{26}H_{35}D_2N_5O_6$.

3-(5-*Acetamido*-6-*hydroxy*-2-*oxo*-1,2-*dihydro*-3-*pyridylimino*)-5-*acetamido*-6-*hydroxy*-2-*pyridone* (17).—A solution of 3-acetamido-5-phenylazocitrazinic acid (3.2 g) in 2M-sodium hydroxide (15 ml) containing W-2 type Raney nickel (5 g) was hydrogenated at 20 °C for 8 h. The catalyst was removed and the solution acidified; the precipitate was very susceptible to oxidation (even under nitrogen) and was dried and then heated on a steam-bath for 15 min, in acetic acid (25 ml). After 1 h, at room temperature, the blue crystalline product (17) was purified by extraction with acetone, m.p. >300 °C (*cf.* ref. 3) (Found: C, 47.9; H, 4.0; N, 19.5%; M^+ 347. Calc. for $C_{14}H_{13}N_5O_6$: C, 48.4; H, 3.8; N, 20.2%; *M* 347). The n.m.r. spectrum (CF_3CO_2H) exhibited signals at τ 7.46 (s, 2 × $OCCH_3$, 6 H) and 0.8 (s, $-C-H$, 2 H); e.s.r. $g = 2.004$. Oxidation of this amine (0.2 g) with chromic oxide gave 3-*acetamidopyridine*-2,5,6-*trione* (0.2 g) which formed plates, m.p. 240—243 °C (decomp.) from acetone–ether (1 : 1) (Found: C, 46.1; H, 3.6; N, 15.6%; M^+ 182. $C_7H_6N_2O_4$ requires C, 46.2; H, 3.3; N, 15.4%; *M* 182); $\lambda_{max.}$ (ethanol) 210 (log ε 4.10), 262 (4.13), and 348 nm (3.76): the n.m.r. spectrum (CD_3COCD_3) exhibited peaks at τ 7.7 (s, $COCH_3$, 3 H), and 2.25 (s, $=C-H$, 1 H).

The amine (17) (0.25 g) was refluxed for 20 h in acetic acid (10 ml) to yield, on cooling, bronze plates, m.p. > 300 °C of 5,5′-*diacetamido*-6,6′-*dihydroxy*-3,3′-*bipyridylidene*-2,2′-*quinone* (0.2 g) (Found: C, 51.0; H, 3.7; N, 16.4%; M^+ 332. $C_{14}H_{12}N_4O_6$ requires C, 50.6; H, 3.6; N, 16.9%; *M* 332): $\lambda_{max.}$ 500 nm (log ε 4.38) in acetic acid: the n.m.r. spectrum (CF_3CO_2H) exhibited signals at τ 7.5 (s, 2 × $OCCH_3$, 6 H) and −0.28 (s, 2 × $=C-H$, 2 H). Knackmuss [3] mentions this quinone, but records no relevant data.

The Acetyl Analogue (25) *of Lemonnierin.*—(a) A solution of 3-acetamido-5-phenylazocitrazinic acid (3.16 g) was hydrogenated as previously described. After removal of the catalyst (under nitrogen) the filtrate was treated with a solution of ferric chloride (0.8 g) in water (5 ml): the mixture was immediately acidified with 20% hydrochloric acid (7 ml) and stirred for 4 h. The temperature was then raised to 35—40 °C and maintained for 20 h. The products were then isolated as for the oxidation of the 3-n-octanoyl analogue to give (i) 3-acetamidopyridine-2,5,6-trione (0.15 g) and (ii) the *acetyl analogue* of lemonnierin (0.5 g) as a microcrystalline, blue solid, m.p. >300 °C after extraction (Soxhlet) with acetone (Found: C, 48.0; H, 3.9; N, 19.7%; M^+ 347. $C_{14}H_{13}N_5O_6$ requires C, 48.4; H, 3.8; N, 20.2%; *M* 347): $\lambda_{max.}$ (acetic acid) 630 nm (log ε 4.75): τ (CF_3CO_2H) 7.60 (s, 2 × $COCH_3$, 6 H) and 7.48 (s, 2 × $-C-H$, 2 H); e.s.r. spectrum, $g = 2.004$.

(*b*) Cyclisation of the amine (17) (0.075 g) in refluxing pyridine (8 ml) containing cupric acetate (0.008 g) during 1¼ h, gave the acetyl analogue of lemonnierin (0.06 g), identical with that prepared by method (a).

Oxidation of this acetyl analogue (25) (0.5 g) with chromic oxide as for lemonnierin gave 3-acetamidopyridine-2,5,6-trione (0.5 g) identical with a previously obtained specimen.

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