Biosynthesis of Santiaguine in *Adenocarpus foliosus*. Part II[1]

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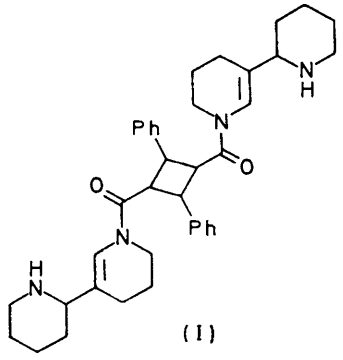
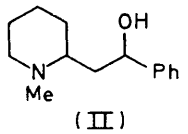
Lysine is shown, by tracer studies on intact *Adenocarpus foliosus* plants, to be incorporated unsymmetrically into the αβ'-bipyridyl unit of the cyclobutane-1,3-dicarbonylbis(decahydrobipyridyl) derivative santiaguine (I). The incorporation proceeds *via* 2,3,4,5-tetrahydropyridine. The acidic portion of santiaguine, α-truxillic acid (*trans*-2,*cis*-4-diphenylcyclobutane-1,*trans*-3-dicarboxylic acid), is shown to be present in *A. foliosus* from feeding experiments with labelled cinnamic acid and is shown to be incorporated into santiaguine. The dimerization of adenocarpine[1-cinnamoyl-1,2,3,4-tetrahydro-5-(2-piperidyl)pyridine] to santiaguine is shown to be enzymically controlled.

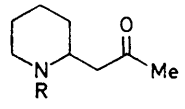
THE alkaloid santiaguine (I) was first isolated in 1950 from *Adenocarpus complicatus Gay*.[2] It has been shown to occur, together with adenocarpine, in other *Adenocarpus* species.[3] We have previously demonstrated that



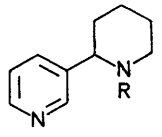
(I)



(II)



(III) R = Me
(IV) R = H

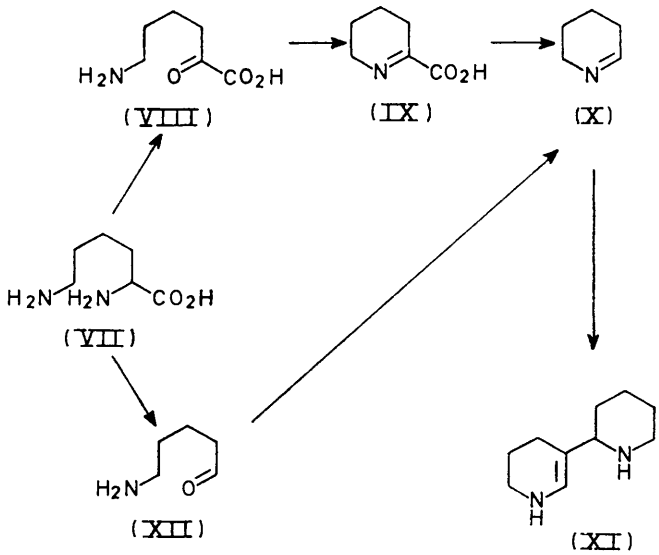


(V) R = H
(VI) R = Me

the α-truxyl (2,4-diphenylcyclobutane-1,3-dicarbonyl) unit of santiaguine is derived from phenylalanine and cinnamic acid and that adenocarpine [1-cinnamoyl-1,2,3,4-tetrahydro-5-(2-piperidyl)pyridine] is the immediate precursor of santiaguine.[1]

It has been reported that labelled cadaverine (pentane-1,5-diamine) is incorporated into the αβ'-bipyridyl portion of santiaguine.[4] The involvement of cadaverine in the normal biosynthesis of the αβ'-bipyridyl ring system, however, is doubtful since although cadaverine is incorporated into the piperidine ring of a number of alkaloids, *e.g.* sedamine (II),[5] *N*-methylisopelletierine (III),[6] and anabasine (V),[7] the incorporation has been shown to occur by an aberrant pathway. These alkaloids, as well as others, are derived unsymmetrically from lysine *via* 2,3,4,5-tetrahydropyridine.[8,9] Further it has been shown that the nitrogen atom at C-6 in lysine furnishes the nitrogen atom of the piperidine ring in anabasine (V)[10] and that both C-6 protons in the amino-acid are retained in sedamine (II)[11] and isopelletierene (IV),[8] indicating that oxidation takes place at C-2 of lysine in the biosynthesis of these alkaloids. These results are consistent with the biosynthetic pathway for tetrahydroanabasine (XI) shown in Scheme 1. Lysine (VII) on transamination yields 6-amino-2-oxohexanoic acid (VIII), which cyclizes to 2,3,4,5-tetrahydropyridine-2-carboxylic acid (IX). Decarboxylation yields tetrahydropyridine (X), which dimerizes.

Recently it has been reported that [2-^{3}H, 6-^{14}C]lysine is incorporated into sedamine (II), *N*-methylisopelletierene (III), and ababasine (V) with unchanged isotopic ratio.[12] To accommodate the previously reported unsymmetrical incorporation of lysine into these alkaloids,

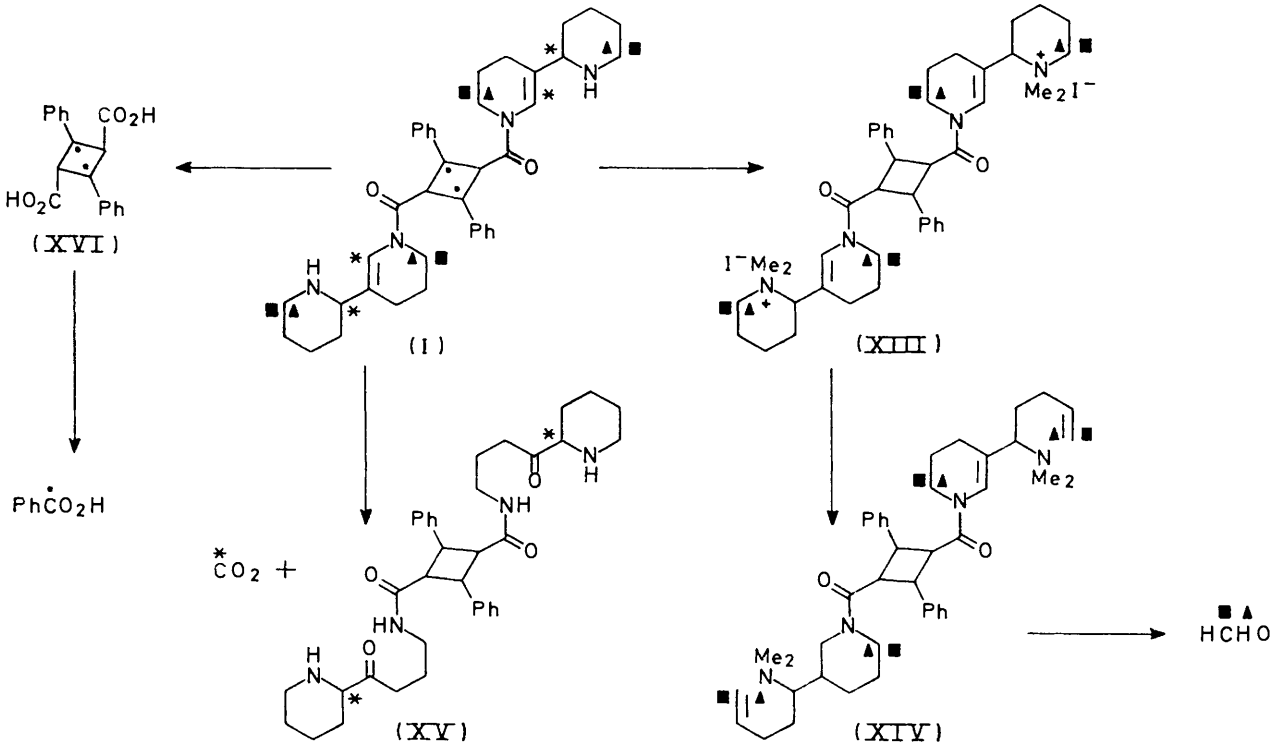


SCHEME 1

methylation of the ε-nitrogen atom of lysine was proposed as the first step in the biosynthesis. More recently in tracer experiments with *Nicotiana tabacum*

[1] D. G. O'Donovan and P. B. Creedon, *J. Chem. Soc.* (*C*), 1971, 1604, is considered as Part I.
[2] I. Ribas and P. Taladrid, *Anales real Soc. espaň Fis. Quim.*, 1950, **46B**, 489.
[3] I. Ribas and L. Costa, *Ann. Pharm. franç.*, 1952, **10**, 54; M. R. Mendez and I. Ribas, *Anales real Soc. espaň. Fis. Quim.*, 1958, **54B**, 157; I. Ribas and M. Ribas, *ibid.*, 1966, **62B**, 845; R. Bernasconi and E. Steinegger, *Pharm. Acta Helv.*, 1970, **45**, 42.
[4] H. R. Schutte, K. L. Kelling, D. Knofel, and K. Mothes, *Phytochem.*, 1964, **3**, 249.
[5] R. N. Gupta and I. D. Spenser, *Canad. J. Chem.*, 1967, **45**, 1275.
[6] M. F. Keogh and D. G. O'Donovan, *J. Chem. Soc.* (*C*), 1970, 1792.
[7] E. Leete, *J. Amer. Chem. Soc.*, 1956, **78**, 3520.
[8] D. G. O'Donovan and L. F. Buckley, unpublished work.
[9] E. Leete, *J. Amer. Chem. Soc.*, 1969, **91**, 1697.
[10] E. Leete, E. G. Gros, and T. G. Gilbertson, *J. Amer. Chem. Soc.*, 1964, **86**, 3907.
[11] R. N. Gupta and I. D. Spenser, *J. Biol. Chem.*, 1969, **244**, 88.
[12] R. N. Gupta and I. D. Spenser, *Phytochem.*, 1970, **9**, 2329.

and *N. gluca* it has been shown that on feeding labelled 2,3,4,5-tetrahydro-*N*-methylpyridinium chloride, active anabasine (V) and *N*-methylanabasine (VI) are formed. However when labelled lysine, an established precursor, was fed to these species no activity was detected in the *N*-methylanabasine obtained, showing that *N*-methylanabasine is not an intermediate in the biosynthesis of anasabine from lysine.[13] Retention of tritium at C-2 could be accounted for by a concerted decarboxylation and oxidation, *via* pyridoxal, to yield 5-aminopentanal (XII). Cyclization yields tetrahydropyridine (X) which then dimerizes to tetrahydroanabasine (XI).



SCHEME 2

To investigate the biosynthesis of the αβ-bipyridyl unit of santiaguine the following feeding experiments were undertaken. (±)-[2-^{14}C]- and (±)-[6-^{14}C]-lysine were fed, in separate experiments to *A. foliosus* plants, and the plants were grown on for 3 weeks before harvesting. Active santiaguine was isolated as described previously. Percentage incorporation figures are reported in Table 1. The active alkaloid from both experiments was degraded as outlined in Scheme 2. Santiaguine (I) was converted to its di-*N*-methyl dimethiodide (XIII), which was then subjected to a Hoffmann degradation yielding a mixture of products including (XIV). Oxidation of (XIV) with osmium tetraoxide (to the diol) followed by periodate gave formaldehyde, isolated as its dimedone derivative. Another sample of the active santiaguine from each feeding experiment was oxidised with acidic permanganate to carbon dioxide, isolated as barium carbonate, and the amide (XV), isolated as its chloroplatinate. Specific activities, reported in Table 2, of santiaguine and its degradation products, in both experiments, show that lysine is incorporated unsymmetrically into both rings of the αβ'-bipyridyl portion of santiaguine.

In a third experiment 2,3,4,5-tetrahydro[2-^{14}C]-pyridine was fed to one *A. foliosus* plant. The plant was grown on for 3 weeks and then harvested. Active santiaguine was isolated as before. The percentage incorporation is reported in Table 1. Degradation of the active santiaguine was effected as in the lysine feeds; the concordance between the specific activities of santiaguine and its degradation products (Table 2) shows that the tetrahydropyridine is incorporated unsymmetrically. The precise nature of the intermediates

TABLE 1

Incorporation of tracers into santiaguine and α-truxillic acid

Tracer	Amount fed (mCi)	Activity (disint. min^{-1} mmol^{-1} × 10^{-5}) Sangiaguine	α-Truxillic acid
(±)-[2-^{14}C]Lysine	0·1	5·98	
(±)-[6-^{14}C]Lysine	0·1	9·12	
2,3,4,5-Tetrahydro-[2-^{14}C]pyridine	0·02	12·3 [a]	
[β-^{14}C]Cinnamic acid	0·1	35·5 [b]	
[β-^{14}C]Cinnamic acid	0·1		3·29
[2,4-^{14}C]-α-Truxilic acid	0·01	6·28	

[a] Specific activity of tetrahydroanabasine unit of santiaguine. [b] Specific activity of α-truxyl unit of santiaguine.

[13] E. Leete and M. R. Chedekel, *Phytochem.*, 1972, **11**, 2751.

between lysine and tetrahydropyridine in the biosynthesis of santiaguine remain to be elucidated. Our results confirm, as in the case of other piperidine alkaloids studied, that cadaverine is not an intermediate in the biosynthesis of santiaguine from lysine.

A. foliosus, [β-^{14}C]cinnamic acid was fed to the two-year-old plant used in the labelled tetrahydropyridine feed 24 h after the tetrahydropyridine had been fed. Active truxillic acid was isolated along with active santiaguine. The incorporation of cinnamic acid into

TABLE 2

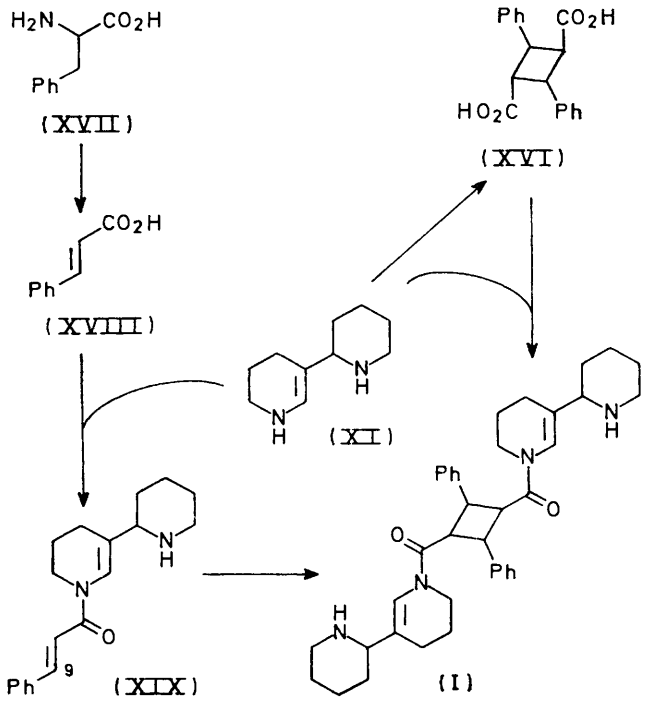
Activities * of santiaguine and its degradation products

	Precursor			
Compound	[2-^{14}C]Lysine	[6-^{14}C]Lysine	Cinnamic acid +2,3,4,5-tetrahydropyridine	α-Truxillic acid
Santiaguine	5·98	9·12	61·2	6·28
α-Truxillic acid	0	0	35·5	6·20
Benzoic acid			17·6	3·12
Tetrahydroanabasine	3·00	4·50	12·3	0
Di-*N*-methylsantiaguine dimethiodide	5·91	9·04	61·7	
Formaldehyde dimedone	0	2·12	5·8	
Barium carbonate	1·31	0	0	
Oxidised amide (XV)	3·09	9·00	60·1	

Activities * of α-truxillic acid and its degradation product: α-truxillic acid 0·329, benzoic acid 0·163

* In disint. min^{-1} mmol^{-1} × 10^{-5}.

Our previous work established (Scheme 3) that phenylalanine (XVII) and cinnamic acid (XVIII) are incorporated specifically into santiaguine and that adenocarpine (XIX) is the immediate precursor of santiaguine (I). The possibility still remains however



SCHEME 3

that α-truxillic acid (XVI) is formed by the dimerization of cinnamic acid. Santiaguine could then arise through amide formation between the acid (XVI) and tetrahydroanabasine (XI). Free α-truxillic acid has never been isolated from plant sources though it occurs in plants as a disubstituted α-truxyl derivative in ester or amide form.[14]

To establish the presence of free α-truxillic acid in santiaguine in this experiment (0·55%) was 10 times as high as previously found. This may be attributable to stimulation of alkaloid biosynthesis by the presence of both precursors necessary for alkaloid formation.

Degradation of the active α-truxillic acid as in Scheme 2, to benzoic acid, showed that the incorporation of cinnamic acid was specific. Percentage incorporations and specific activities of α-truxillic acid and its degradation product, benzoic acid, are reported in Tables 1 and 2.

To confirm that α-truxillic acid is incorporated into santiaguine [2,4-^{14}C]-α-truxillic acid, synthesised from *trans*-[β-^{14}C]cinnamic acid,[1] was fed to two one-year-old *A. foliosus* plants. The plants were allowed to grow on for 21 days, then harvested, and active santiaguine was isolated as before. Degradation of the active santiaguine showed that α-truxillic acid is specifically incorporated into the alkaloid.

Our results establish that α-truxillic acid occurs in *A. foliosus* and that is formed in the plant from cinnamic acid. Furthermore α-truxillic acid is specifically incorporated into santiaguine. Thus two distinct biosynthetic pathways to santiaguine appear to exist in *A. foliosus* as outlined in Scheme 3, the major one involving adenocarpine as an intermediate. However, the relative incorporations (*a*) of cinnamic acid into α-truxillic acid (0·02%) and santiaguine (0·55%) and (*b*) of adenocarpine (3·30%) and α-truxillic acid (0·96%) into santiaguine could be interpreted as indicating that there is only one route to santiaguine (*via* adenocarpine); and that α-truxillic acid arises through an equilibrium that may exist between the amide, santiaguine, and its components α-truxillic acid and tetrahydroanabasine. The results presented here do not allow us to distinguish between these two possibilities.

It has been established that α-truxillic acid is formed

[14] K. Mothes and H. R. Schutte, ' Biosynthese der Alkaloide,' UEB Deutscher Verlag der Wissenschaften, Berlin, 1969, p. 183; M. Quadrat-i-Khuda, M. Erfan Ali, and L. A. M. Samsuzzaman, *J. Sci. Res.* (*Pakistan*), 1965, **2**, 8.

from *trans*-cinnamic acid by specific photochemical processes.[15] The possibility therefore arises that the dimerization of adenocarpine to give santiaguine may be photochemically controlled *in vivo* in *A. foliosus*.[16]

To test this hypothesis the following tracer experiments were undertaken. One six-month-old *A. foliosus* plant was kept in complete darkness for 24 h. [9-^{14}C]-Adenocarpine, synthesized as described previously, was then fed to the plant, which was grown on for 3 days. The plant was harvested and active santiaguine was isolated, incorporation 0·17%. Simultaneously [9-^{14}C]-adenocarpine was administered to a comparable six-month-old plant growing normally under greenhouse conditions. The plant was grown on for 3 days, then harvested, and active santiaguine was isolated, incorporation 0·30%. The discrepancy in incorporation of tracer can be ascribed to the better growing conditions enjoyed by the plant grown under greenhouse conditions. These results show that the dimerization of adenocarpine to santiaguine is enzymically rather than photochemically controlled.

EXPERIMENTAL

M.p.s are corrected. Radioactive assays were carried out with a Nuclear Chicago Unilux II liquid scintillation counter and results were processed by an off-line Olivetti P101 computer, corrections being made for quenching and background. Standard scintillator solvents were used throughout.

Administration of Tracers to A. foliosus *and Isolation of Santiaguine.*—In separate experiments [2,4-^{14}C]-α-truxillic acid (total activity 0·01 mCi), (±)-[6-^{14}C]lysine (total activity 0·1 mCi), and (±)-[2-^{14}C]lysine (total activity 0·1 mCi) were fed, by a wick arrangement, to two one-year-old *A. foliosus* plants. The plants were allowed to grow on for 3 weeks and active santiaguine was isolated as described previously.[1]

In another experiment 2,3,4,5-tetrahydro[2-^{14}C]pyridine (total activity 0·02 mCi) and [β-^{14}C]cinnamic acid (total activity 0·1 mCi) were fed, with a 24 h interval, to one two-year-old *A. foliosus* plant. A homogenate was prepared as before. Inactive santiaguine (200 mg) and inactive truxillic acid (400 mg) were added as carriers. After acidification of the ethanolic concentrate, the mixture was extracted with chloroform (4 × 50 ml) and the combined extracts were concentrated to *ca.* 40 ml and was extracted with 3% sulphuric acid (2 × 20 ml). The aqueous layer was combined with the previous one and the combined acidic solution was worked up to yield santiaguine (190 mg). The dried (Na_2SO_4) chloroform solution was evaporated to dryness under reduced pressure. The brown semi-solid remaining was extracted with ether (5 × 20 ml) and the resulting white powder was recrystallized 3 times from 95% ethanol yielding α-truxillic acid (307 mg), m.p. and mixed m.p. 281—282° (lit.,[15] 283—284°).

In separate experiments [9-^{14}C]adenocarpine (total activity in each case 0·02 mCi) was fed simultaneously to two six-month-old *A. foliosus* plants, one growing in a greenhouse, and the other in darkness (in a photographic darkroom). Both plants were grown on for 3 days, then harvested, and santiaguine was isolated as before. The plant grown in the darkroom was worked up to the final stage under exposure to a red photographic safety light only.

Degradation of Santiaguine.—(a) [2-^{14}C]*Lysine feed. Permanganate oxidation.* The active santiaguine (100 mg) was dissolved in a mixture of sulphuric acid (0·1 ml) and water (1 ml). The flask was connected to a gas train fitted with a permanganate–sulphuric acid scrubber and the system was flushed with pure nitrogen. Two carbon dioxide traps, containing freshly prepared barium hydroxide, were added to the train. To the reaction mixture was added, slowly and with stirring, an aqueous solution of chromic anhydride (250 mg) and sulphuric acid (0·2 ml). The flask was heated on a water-bath for 3 h and then allowed to cool. The system was flushed with pure nitrogen for a further 2 h. The precipitated barium carbonate (60 mg) was filtered off, washed with ethanol and ether, and dried. The reaction mixture was filtered and brought to pH 7 with barium hydroxide. The mixture was again filtered to remove inorganic salts and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water (1 ml) and aqueous chloroplatinic acid (1 ml) was added. A brown solid separated. It was filtered off, washed with ice-cold water, and dried (desiccator) to yield the *chloroplatinate* of 1,3-bis-[4-oxo-4-(2-piperidyl)butylcarbamoyl]-2,4-diphenylcyclobutane (XIII) (52 mg), m.p. 240—243° (decomp.) (Found: C, 42·5; H, 4·95; N, 5·3. $C_{36}H_{50}Cl_6N_4O_4Pt$ requires C, 42·75; H, 5·0; N, 5·55%).

(b) [6-^{14}C]*Lysine feed. Di-*N*-methylsantiaguine dimethiodide.* Sodium (25 mg) was added to liquid ammonia (15 ml) containing a crystal of iron(III) nitrate. Santiaguine (110 mg) in ether (20 ml) was added with stirring. After 10 min methyl iodide (0·4 ml) was added and the solution was stirred for 10 min. The ammonia was allowed to evaporate, water (10 ml) and chloroform (20 ml) were added with shaking, and the organic layer was separated, dried (Na_2SO_4), and evaporated. The residue was refluxed in methanol (10 ml) and methyl iodide (0·5 ml) for 8 h. The solution was evaporated to small bulk and diluted with ether. The methiodide (123 mg), m.p. 185—186°, separated as a fine yellow solid.

Hoffmann degradation of the methiodide and oxidation of the product. The methiodide (115 mg) was dissolved in 80% ethanol (8 ml) and an excess of freshly prepared moist silver oxide was added. The mixture was stirred for 40 min and filtered. The filtrate was evaporated to dryness at 40° under reduced pressure and the residue was distilled (210°; 10^{-3} mmHg) to yield the amide (XIV). This was dissolved in dry ether (20 ml) containing pyridine (0·1 ml). The solution was cooled to −40°, osmium tetraoxide (300 mg) in ether (5 ml) was added, and the mixture was stirred for 30 min. A light brown precipitate formed. The mixture was allowed to come to room temperature and left for 2 h. The osmate ester–pyridine complex was filtered off, washed with ether, and dried.

The complex was added to a solution of sodium sulphite (150 mg) and potassium carbonate (60 mg) in 50% ethanol (6 ml) and the mixture was stirred at room temperature for 1 h. The solution was extracted with chloroform (2 × 10 ml) and the extracts were dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in a little water and added to potassium periodate (150 mg) in water (200 ml).

[15] H. I. Bernstein and W. C. Quimby, *J. Amer. Chem. Soc.*, 1943, **65**, 1845; E. H. White and H. C. Dunathan, *ibid.*, 1956, **78**, 6055; M. B. Hocking, *Canad. J. Chem.*, 1969, **47**, 4567.

[16] L. Costa and I. Ribas, *Anales real Soc. Fis. espaň. Quim.*, 1952, **48B**, 699.

The mixture was distilled and the distillate absorbed in aqueous dimedone (100 ml). Formaldehyde dimedone derivative separated as needles (63 mg), m.p. and mixed m.p. 192—193°.

(c) 2,3,4,5-*Tetrahydro*[2-^{14}C]*pyridine feed*. The santiaguine from this feed was degraded as described above.

(d) [2,4-^{14}C]-α-*Truxillic acid feed*. The santiaguine from this feed was degraded as described previously.[1]

Degradation of α-truxillic acid. α-Truxillic acid was degraded to benzoic acid as described previously.[1]

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