819. *Alkaloid Biosynthesis. Part VI.* The Biosynthesis of Colchicine.*

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Unambiguous degradative methods are devised for the isolation of specific carbon atoms from colchicine. These methods are used in tracer experiments on *Colchicum autumnale* which establish that ring A and carbon atoms 5, 6, and 7 of colchicine are derived from the phenylalanine–cinnamic acid pathway. Tyrosine cannot replace phenylalanine for this part of the molecule.

The tropolone ring is shown not to be derived from acetic acid, in contrast to the mould tropolones. However, the specific incorporation of activity from [3-^{14}C]tyrosine into the tropolone ring is established, and degradation of the active colchicine reduces to four the number of possible sites which could carry this label. On the basis of these results, a scheme for the biosynthesis of colchicine is proposed which makes use of a C_6–C_3 unit and a C_6–C_1 unit joined to give a C_6–C_3–C_6–C_1 system.

Preliminary accounts of the main results have already been published.[1,2]

Colchicum autumnale Linn., the meadow saffron, produces a mixture of alkaloids [3,4] all of which are related structurally to the major alkaloid colchicine (I); mixtures containing colchicine are also present in several other Liliaceae genera.[3,4] Colchicine not only has an unusual structure and valuable biological properties [5] but in addition poses biosynthetic problems of great interest. Thus, one cannot readily suggest which simpler molecules are the ones most probably used by the plant to form the alkaloid. Further, the tropolone residue, rare in natural products from higher plants, might be constructed in several plausible ways, and, finally, a pathway must be envisaged to account for the unusual position of the nitrogen atom. A reflection of these problems is the considerable number of proposals for the biosynthesis of colchicine and of the tropolone nucleus.[6–13] Our aim in the work reported in the present Paper was a determination of the origin of the carbon skeleton of colchicine; this knowledge is the prerequisite of future progress.

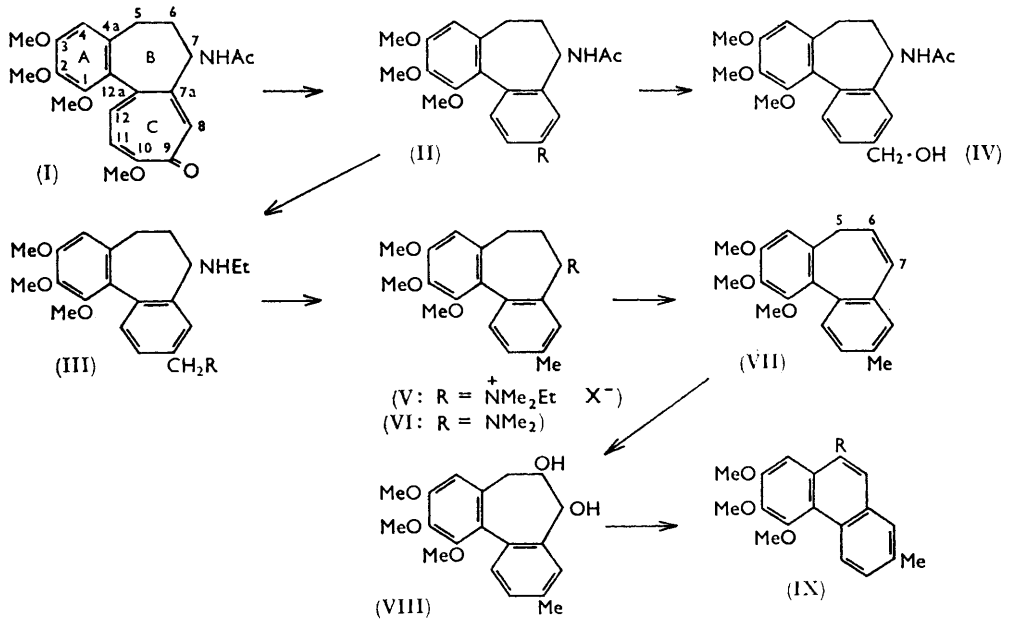
The choice of possible precursors was influenced by Anet and Robinson's proposal [11] that the biosynthesis of colchicine may be related to that of the flavones and similar C_6–C_3–C_6 systems. These had been shown [14] at the outset of our work to be built from a phenylpropanoid unit (Ar–C–C–C) and acetic acid, and so it was reasoned that ring C of colchicine may arise largely from acetic acid and ring A, with carbon atoms 5, 6, and 7 from the phenylpropanoid unit. Thoughts along these lines were encouraged by Bentley's work [15] on the biosynthesis of mould tropolones, which showed the incorporation of activity from

* Part V, *J.*, 1964, 4078.

[1] Battersby and Reynolds, *Proc. Chem. Soc.*, 1960, 346.
[2] Battersby, Binks, and Yeowell, *Proc. Chem. Soc.*, 1964, 86.
[3] Cook and Loudon, "The Alkaloids," ed. Manske and Holmes, Academic Press, New York, 1952, Vol. II; Wildman, "The Alkaloids," ed. Manske, Academic Press, New York, 1960, Vol. VI, p. 247.
[4] Boit, "Ergebnisse der Alkaloid-Chemie bis 1960," Akademie Verlag, Berlin, 1961, p. 28.
[5] Eigsti and Dustin, "Colchicine in Agriculture, Medicine, Biology, and Chemistry," Iowa State College Press, Ames, Iowa, 1955.
[6] Dewar, *Nature*, 1950, **166**, 790.
[7] Raistrick, *Proc. Roy. Soc.*, 1949, *A*, **199**, 141.
[8] Robinson, *Nature*, 1950, **166**, 924.
[9] Erdtman and Todd, Symposium on Tropolones and Allied Compounds, *Chem. and Ind.*, 1951, 12.
[10] Belleau, *Experientia*, 1953, **9**, 178.
[11] Anet and Robinson in Robinson, "The Structural Relations of Natural Products," Clarendon Press, Oxford, 1955, p. 47.
[12] Wenkert, *Experientia*, 1959, **12**, 165.
[13] Scott, *Nature*, 1960, **186**, 556.
[14] *Inter alia*, Watkin, Underhill, and Neish, *Canad. J. Biochem. and Physiol.*, 1957, **35**, 229; Grisebach, *Z. Naturforsch.*, 1957, **12**b, 227; Geissman and Swain, *Chem. and Ind.*, 1957, 984; Shibata and Yamazaki, *Pharm. Bull.* (*Japan*), 1957, **5**, 501.
[15] Bentley, *Biochem. Biophys. Acta*, 1958, **29**, 666.

sodium [1-^{14}C]acetate into stipitatic acid. Accordingly, aqueous solutions of (±)-[2-^{14}C]-tyrosine and sodium [1-^{14}C]acetate were injected separately into the seed capsules of *C. autumnale* plants, and the colchicine isolated from both groups of plants was radioactive (Table 1).

If the foregoing ideas are broadly correct, activity from [2-^{14}C]tyrosine should be concentrated largely at position 6 of colchicine, and a degradation of the alkaloid was developed to isolate this carbon atom. Trial experiments on colchicine itself showed that it would be advantageous to convert the tropolone system into a stable benzenoid nucleus. Consequently, colchicine was converted by the method of Fernholtz [16] into allocolchiceine (II; R = CO_2H), a rearrangement which was known [16,17] to occur unambiguously with the extrusion of carbon atom 9. Methylation with diazomethane then afforded allocolchicine [16] (II; R = CO_2Me). This could be reduced with lithium aluminium hydride to give, depending upon the conditions, a mixture containing mainly neutral material or mainly basic products. The alcohol (IV) was isolated from the neutral fraction and the amine (III; R = OH) from the basic one. Careful study of this reduction allowed high and reproducible yields of the base (III; R = OH) to be obtained. Hydrogenolysis of the benzylic alcohol residue in this material proceeded smoothly over palladium in the presence of perchloric acid to afford the base (III; R = H). This now had the required stability to allow Hofmann elimination to be attempted, and the first stage, involving conversion into the quaternary iodide (V; X = I), was accomplished with methyl iodide and potassium carbonate or with alkaline dimethyl sulphate followed by the addition of an excess of potassium iodide.



It was expected on the basis of Ingold's studies [18] that elimination to afford the conjugated olefin (VII) would be favoured over loss of the less-substituted ethylene. In the event, Hofmann degradation of the quaternary hydroxide (V; X = OH) under rather vigorous conditions gave a mixture containing about 70% of neutral material, the remainder

[16] Fernholtz, *Annalen*, 1950, **568**, 63; Santavy, *Helv. Chim. Acta*, 1948, **31**, 821; Lettre, *Angew. Chem.*, 1947, **59**, 218.
[17] Doering and Denney, *J. Amer. Chem. Soc.*, 1955, **77**, 4619.
[18] Hanhart and Ingold, *J.*, 1927, 997; Hughes, Ingold, and Maw, *J.*, 1948, 2072; Hughes, Ingold, Maw, and Woolf, *J.*, 1948, 2077.

being composed of quaternary substances and a base. The last afforded a crystalline picrate of composition corresponding to structure (VI); this assignment was supported by quaternisation of the base with methyl iodide followed by Hofmann elimination to afford the same neutral product which had been obtained above.

Careful chromatography of the neutral product derived from the salt (V) showed it to be a mixture, though it was only possible to enrich the components in certain fractions. Hydrogenation of two fractions which differed considerably in melting point yielded the same dihydro-material (as VII, double-bond reduced) thus showing that the original mixture contained the expected product (VII) together with its isomer having the olefinic residue in the 5,6-position. Other workers have observed this migration towards the alkoxylated ring in related cases.[19] When the Hofmann degradation was carried out under milder conditions, only a little of the 5,6-isomer was formed and the crystalline cycloheptatriene (VII) was isolated. Subsequent steps were based upon the experiments of Cook and his co-workers [19] with a related series of compounds. Osmium tetroxide oxidation of the olefin (VII) gave the *cis*-diol (VIII) which was cleaved by lead tetra-acetate to a dialdehyde. This, without purification, was cyclised by sodium carbonate to the yellow aldehyde (IX; R = CHO). Controlled oxidation by permanganate then afforded the phenanthrenecarboxylic acid (IX; R = CO_2H) which was smoothly decarboxylated over copper chromite

TABLE 1.

Tracer experiments on *Colchicum autumnale*.

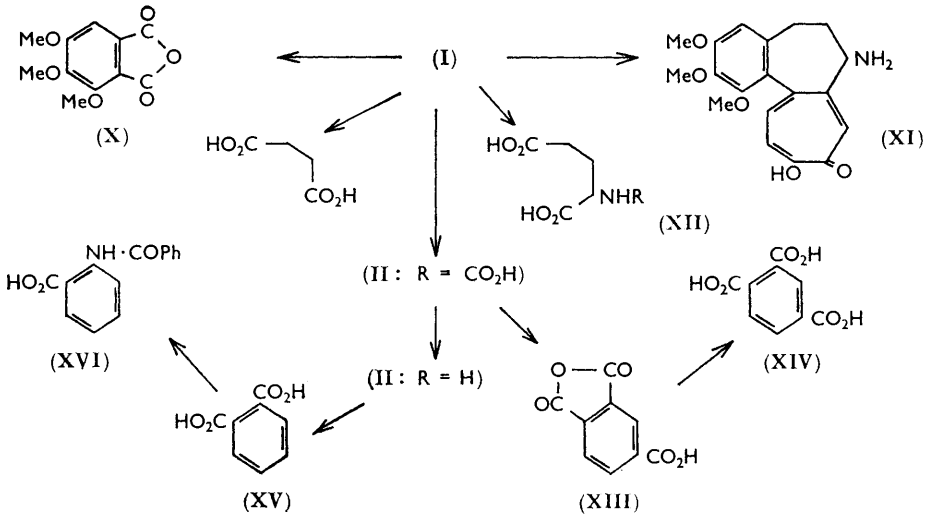
Precursor	No. of plants	Year	Wt. of colchicine (mg.)	Incorporation (%)
0·023 mc (±)-[2-^{14}C]Tyrosine	3	1958	45	0·21
0·2 mc (±)-[2-^{14}C]Tyrosine	6	1959	187	0·23
0·15 mc Sodium [1-^{14}C]acetate	3	1958	40	0·19
2·0 mc Sodium [1-^{14}C]acetate	8	1959	223	0·13
0·016 mc L-[Me-^{14}C]Methionine	2	1959	30	0·90
0·1 mc [CO_2H-^{14}C]Benzoic acid	2	1961	32	$<5 \times 10^{-4}$
0·2 mc [CO_2H-^{14}C]Protocatechuic acid	4	1961	56	$<4 \times 10^{-3}$
0·2 mc [2-^{14}C]Glycine	3	1961	46	0·8
0·1 mc Sodium [2-^{14}C]pyruvate	3	1961	50	0·25
0·1 mc (±)-[2-^{14}C]Phenylalanine	7	1962	83	0·58
0·1 mc (±)-[1-^{14}C]Phenylalanine	10	1963	312	3·2
0·1 mc (±)-[3-^{14}C]Tyrosine	10	1962	196	0·35
0·2 mc (±)-[3-^{14}C]Tyrosine	19	1963	398	0·7
0·15 mc Sodium [2-^{14}C]cinnamate	10	1963	266	0·22
0·13 mc Sodium [3-^{14}C]cinnamate	10	1962	242	1·0
0·1 mc (±)-[1-^{14}C]Tyrosine	6	1962	80	6×10^{-3}

to give the phenanthrene (IX; R = H) and carbon dioxide corresponding to position 6 of the original colchicine. The carbon dioxide was collected as barium carbonate. This method of degradation [1] has recently been applied to demecolcine.[20]

When the radioactive colchicine from the experiment with sodium [1-^{14}C]acetate was taken through this degradation, the cycloheptatriene (VII) contained little activity (Table 2) whereas the allocolchiceine (II; R = CO_2H) from which it was derived retained all the activity of the original colchicine. It follows that almost all the activity of the colchicine is concentrated in the *N*-acetyl group, and the remaining activity was found to be in ring A and its attached carbon atoms by oxidation of the alkaloid to 3,4,5-trimethoxyphthalic anhydride (X) (Table 2). A further portion of the allocolchiceine (II; R = CO_2H) was oxidised [16] to trimellitic acid (XIV) which is conveniently isolated as the anhydride (XIII). The derived acid (XIV) was not radioactive.

These results are interpreted as proof that the tropolone nucleus of colchicine is not derived from acetate units, in contrast to the mould tropolones.[15,21] Leete and Nemeth [22]

[19] Barton, Cook, and Loudon, *J.*, 1945, 176.
[20] Leete, *J. Amer. Chem. Soc.*, 1963, **85**, 3666.
[21] Richards and Ferretti, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 107.
[22] Leete and Nemeth, *J. Amer. Chem. Soc.*, 1960, **82**, 6055; *ibid.*, 1961, **83**, 2192.



carried out concurrent experiments with labelled acetic acid on a different species (*C. byzantinum*) and their results are in agreement with ours.

Degradation of the colchicine from the experiment with [2-^{14}C]tyrosine was taken through to the phenanthrene (IX; R = H) and carbon dioxide; the results (Table 2) establish scatter of the label. Approximately 50% of the original activity was present in

TABLE 2.

Degradation of radioactive colchicine.

Colchicine and degradation products	Precursors and rel. activity: [2-^{14}C]-Tyrosine	Sodium [1-^{14}C]acetate
Colchicine (I)	1·00	1·00
Allocolchiceine (II; R = CO_2H)	0·91	1·00
Cycloheptatriene (VII)	0·39	0·04
Phenanthrenealdehyde (IX; R = CHO)	0·39	—
Trimethoxymethylphenanthrene (IX; R = H)	0·36	—
Carbon dioxide from (IX; R = CO_2H)	0·01	—
Trimethoxyphthalic anhydride (X)	—	0·04
Trimellitic acid (XIII)	—	0·00
Carbon dioxide from (II; R = CO_2H)	0·00	—
N-Phenylphthalimide	0·01	—

	[3-^{14}C]-Tyrosine
Colchicine (I)	1·00
Trimethoxyphthalic anhydride (X)	0·02
Allocolchiceine (II; R = CO_2H)	1·00
Carbon dioxide from (II; R = CO_2H)	0·00
Deacetylcolchiceine (XI)	0·89
p-Bromophenacyl acetate	0·13
Di-*p*-bromophenacyl succinate	0·03
N-Acetylglutamic acid	0·15
Glutamic acid *N*-phenylurea	0·02
N-Phenylphthalimide	0·81
N-Benzoylanthranilic acid	0·81

Colchicine and degradation products	Precursors and rel. activity: [1-^{14}C]-Phenylalanine	[2-^{14}C]-Phenylalanine	[3-^{14}C]-Cinnamic acid	[2-^{14}C]-Cinnamic acid	[1-^{14}C]-Tyrosine
Colchicine (I)	1·00	1·00	1·00	1·00	1·00
Deacetylcolchiceine (XI)	1·04	1·01	1·00	—	0·79
Trimethoxyphthalic anhydride (X)	0·05	0·05	1·00	0·05	0·60
Di-*p*-bromophenacyl succinate	0·99	—	1·02	1·06	—
CO_2 from Schmidt on succinic acid	1·01	—	0·03	0·03	—

the *N*-acetyl group, whereas the carbon isolated as carbon dioxide from position 6 carried only 1%. A further degradation started with allocolchiceine (II; R = CO_2H) which was decarboxylated over copper chromite in boiling quinoline to afford decarboxyallocolchiceine (II; R = H) and inactive carbon dioxide. Oxidation of the former then afforded phthalic acid (XV) which was isolated as its *N*-phenylimide; this was almost inactive (Table 2). Clearly tyrosine, or its close relatives, had not been incorporated in the way envisaged above and it seemed probable that cleavage had occurred in the plant to produce a non-radioactive C_6–C_1 unit together with a labelled C_2-unit. The latter could undergo further modification to cause labelling of the acetate and one-carbon unit pools. This view was reinforced when Leete and Nemeth [22] reported that [3-^{14}C]phenylalanine fed to *C. byzantinum* plants gave rise to colchicine which carried 93% of the original activity at position 5. Accordingly, we fed labelled C_6–C_1 substances (benzoic acid, 3,4-dihydroxybenzoic acid) to *C. autumnale* plants, and other more speculative feeding experiments were carried out at that time with [2-^{14}C]glycine and sodium [2-^{14}C]pyruvic acid. These were taken only as far as a determination of the incorporation values (Table 1) because results from other studies outlined below caused us to question this interpretation and to carry out appropriate experiments.

Tracer experiments on *Amaryllidaceae* alkaloids showed [23] that these bases are derived from precursors of the type Ar–C–C–N(R)–C–Ar. The C_6–C_2 unit of this system is derivable from tyrosine but not from phenylalanine, whereas the reverse holds for the C_6–C_1 unit; the two sets of precursors are on quite separate pathways.[24–26] When this knowledge is combined with the foregoing results a new possibility can be considered, that ring A and carbon atoms 5, 6, and 7 of colchicine could be derived from phenylalanine, and the remaining C_7 unit could be an expanded C_6–C_1 unit arising, perhaps, from tyrosine by fission.

The first part of this idea was tested by feeding (±)-[1-^{14}C]phenylalanine and (±)-[2-^{14}C]phenylalanine to *C. autumnale* plants. Also, since phenylpropanoid units are commonly introduced into natural products by way of cinnamic acid,[27] [3-^{14}C]cinnamic acid and [2-^{14}C]cinnamic acid were fed as the sodium salts to autumn crocus plants. The former acid had been prepared earlier [25] and the latter resulted from Knoevenagel condensation [28] of benzaldehyde with [2-^{14}C]malonic acid. To confirm the result from the experiment above based upon [2-^{14}C]tyrosine, further plants were fed with (±)-[1-^{14}C]-tyrosine. Good incorporations were achieved with all these precursors save the last which, as hoped, was used to a barely significant extent (Table 1).

The highly active colchicine from [1-^{14}C]phenylalanine was degraded by oxidation to the anhydride (X), by hydrolysis to deacetylcolchiceine [29] (XI), and by oxidation with chromic acid to afford succinic acid, as indicated in the scheme. Further degradation of the succinic acid by the Schmidt reaction gave carbon dioxide which was counted by absorption in tetramethylammonium hydroxide solution. The results (Table 2) establish that the original colchicine is labelled specifically at position 7 and that there is negligible scatter of activity. The high incorporation of [2-^{14}C]phenylalanine is in keeping with this result, and the radioactive colchicine was degraded to the anhydride (X) and to base (XI) as in the previous case. The relative activities of these materials (Table 2) are in keeping with specific labelling of the original colchicine at position 6. The incorporation of [2-^{14}C]-phenylalanine without randomisation into colchicine and demecolcine in *C. byzantinum* has been shown recently by Leete,[20] and the full degradation of our material from *C. autumnale* has therefore been deferred.

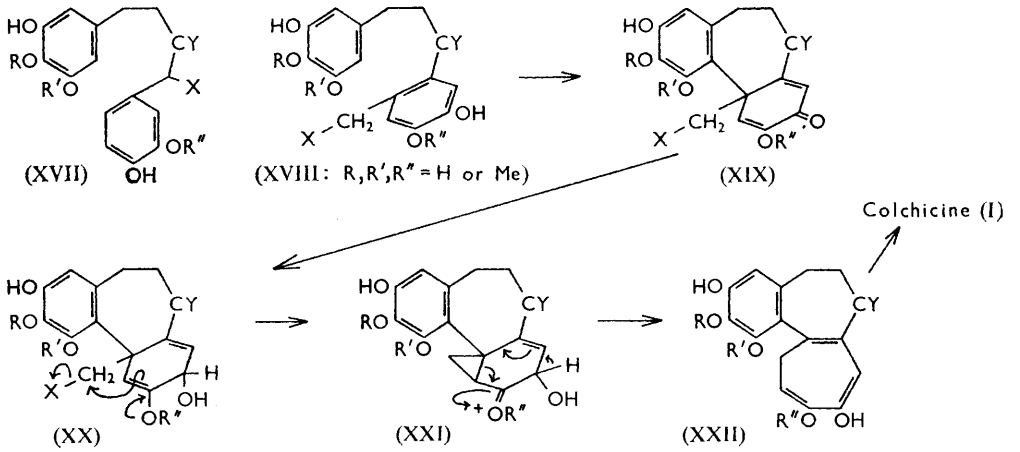
[23] For summaries see Battersby, Tilden Lecture, *Proc. Chem. Soc.*, 1963, 189; Barton, Hugo Muller Lecture, *ibid.*, 1963, 293.
[24] Wildman, Battersby, and Breuer, *J. Amer. Chem. Soc.*, 1962, **84**, 4599.
[25] Battersby, Binks, Breuer, Fales, Wildman, and Highet, *J.*, 1964, 1595.
[26] Suhadolnik, Fischer, and Zulalian, *J. Amer. Chem. Soc.*, 1962, **84**, 4348.
[27] Neish, *Ann. Rev. Plant Physiol.*, 1960, **11**, 55.
[28] Dalal and Dutt, *J. Indian Chem. Soc.*, 1932, **9**, 309.
[29] Zeisel, *Monatsh.*, 1888, **9**, 1.

The colchicine samples from the feeding experiments with [3-^{14}C]-, and [2-^{14}C]-cinnamic acid were degraded as in the foregoing experiment with [1-^{14}C]phenylalanine. The results (Table 2) establish that these precursors lead to colchicine which is specifically labelled at position 5 and at position 6, respectively. The combined strength of the foregoing data leaves no doubt that ring A and carbon atoms 5, 6, and 7 of colchicine are derived from the phenylalanine–cinnamic acid pathway.

In agreement with this conclusion, we find that the weakly active colchicine derived from (±)-[1-^{14}C]tyrosine is not specifically labelled. Removal of the *N*-acetyl and *O*-methyl group (at position 10) causes a considerable fall in activity (Table 2) whilst the derived anhydride (X) carries 60% of the total activity. If the activity were randomly scattered over the molecule, then the anhydride would contain 50% of the total activity.

The second part of the postulated biosynthesis was tested by feeding (±)-[3-^{14}C]tyrosine which gave a good incorporation of activity, and Table 2 collects the degradative results. No significant part of the carbon-14 was present in ring A and its attached carbon atoms as shown by oxidation to the anhydride (X). Hydrolysis to give the base (XI), however, caused the loss of 11% of the total activity, and since there was no change in activity accompanying rearrangement of the original colchicine to allocolchiceine (II; R = CO_2H), there can be no labelling of the *O*-methyl group at position 10. It follows that the *N*-acetyl group contains 11% of the total activity. This is confirmed below.

Vigorous ozonolysis of colchicine by the method of Corrodi and Hardegger [30] gave a mixture of acids from which succinic acid and *N*-acetyl-L-glutamic acid (XII; R = Ac) were isolated by preparative thin-layer chromatography. The activity of the former, isolated as the di-*p*-bromophenacyl ester, shows that carbon atoms 4a, 5, 6, and 7 are very weakly active. The glutamic acid (XII; R = Ac) contained 15% of the total activity, and, knowing that the *N*-acetyl group contains 11%, one can deduce that the α-carboxyl group of this molecule, and so position 7a of colchicine, must contain little or no activity. This was established by hydrolysis of the *N*-acetylglutamic acid and conversion of the resultant amino-acid into the corresponding *N*-phenylurea derivative (XII; R = CO·NHPh) which contained little activity. The acetic acid from this hydrolysis was



converted into its *p*-bromophenacyl ester and the activity of this derivative confirmed the value obtained above by calculation of differences of activity.

The absence of activity at position 7a is important, for this eliminates schemes similar to that outlined below but based upon C_6–C_4–C_6 precursors of the type (XVII).

[30] Corrodi and Hardegger, *Helv. Chim. Acta*, 1955, **38**, 2030.

In summary at this stage, our results show that activity from [3-^{14}C]tyrosine is incorporated to a small extent (11%) into the *N*-acetyl group, no doubt as a result of degradation, and that there is a very low level of scattered activity in much of the carbon skeleton. However, approximately 83% of the total remains to be located and, bearing in mind the foregoing data, this activity must be present in one or more of the five carbon atoms 8 to 12 inclusive. One, at position 9, was readily shown to carry no activity by decarboxylation of allocolchiceine (II; R = CO_2H) as above to afford inactive carbon dioxide. To establish directly that the high level of labelling was indeed present in the tropolone system, oxidation of the decarboxylation product (II; R = H) was carried out to yield phthalic acid (XV). Part was rigorously purified as the corresponding anhydride and as the *N*-phenylimide, and the activity of the latter (Table 2) proved that almost all (97%) of the activity not present in the earlier fragments was retained in the phthalic acid. Schmidt degradation of the phthalic acid gave anthranilic acid, purified as its *N*-benzoyl derivative (XVI). There was no detectable change in activity as a result of this elimination of one carboxyl group, and because of their equivalence in phthalic acid both carboxyl groups are shown to carry little or no activity. The carboxyl groups correspond to the carbons at positions 1a and 7 of colchicine and so these findings interlock with those above.

It is thus proved * that activity from [3-^{14}C]tyrosine is incorporated specifically, apart from minor scatter, into the tropolone ring of colchicine. Only positions 8, 10, 11, and 12 remain as possible sites for this activity. The first clear information is thus available concerning the origin of the tropolone system. The three carbon atoms of the tropolone ring which have been examined (7a, 9, 12a) do not carry a significant amount of scattered activity and it seems unlikely that the remaining four will differ in this respect. The label is expected to be concentrated at one or, at most, two positions.

We propose that the biosynthesis of colchicine involves phenol oxidation of a C_6–C_3–C_6–C_1 precursor of the type (XVIII) built from a C_6–C_3 unit on the phenylalanine–cinnamic acid pathway and a C_6–C_1 unit derivable by fission of tyrosine or a close relative. Minor variations of the scheme are possible; X should, however, be a good leaving group. The suggested ring-expansion step (XX) ⟶ (XXI) ⟶ (XXII) is of the type achieved *in vitro* for simple tropolones by Chapman and Fitton [31] and it is an important part of the scheme that phenol oxidation † leads directly to the required dienone (XIX) and dienol (XX). The importance of dienols in alkaloid biosynthesis has been emphasised elsewhere.[23] If this scheme is followed, colchicine derived from the experiment with [3-^{14}C]tyrosine should be heavily labelled at position 12; discussion of the finer points, *e.g.*, the nature of the groups X, Y, and R, is deferred until further degradative results are available.

Experimental

For general directions and the method used for calculation of incorporations, see Parts III [25] and IV.[32] The methods described there for proof of purity of the isolated alkaloids were also used in the present work. Early assays of radioactivity were carried out as reported in Part II [33] but most of the counting was by the method described in Part III.

Cultivation of Colchicum autumnale *plants and Administration of Labelled Precursors.*—Wild autumn crocus plants were transplanted during the dormant period (July–Aug.) into good loamy soil; seed capsules were produced usually during May. Aqueous solutions of the

* Most of the foregoing results were reported at the I.U.P.A.C. Meeting, London, July 1963. At that time, Professor E. Leete also reported the incorporation of [3-^{14}C]tyrosine into colchicine but it was not known whether the label was present in the tropolone system.

† Professor A. I. Scott has kindly informed us that he proposed a similar scheme based upon a C_6–C_4–C_6 precursor at the 1963 Gordon Conference, U.S.A.

[31] Chapman and Fitton, *J. Amer. Chem. Soc.*, 1963, **85**, 41.
[32] Battersby, Binks, Francis, McCaldin, and Ramuz, *J.*, 1964, 3600.
[33] Battersby, Binks, and Harper, *J.*, 1962, 3534.

various precursors (usually 0·5 ml.) were introduced into the hollow capsules with a fine hypodermic needle and, depending upon the total volume of solution used, two to six such injections were made into each plant during 1—2 weeks. The plants were harvested 4—5 weeks after the start of the feeding experiment.

Extraction and Separation of the Alkaloids.—This method was based upon that reported [34] for the extraction of colchicine from *C. autumnale* seeds. Two whole autumn crocus plants were macerated with ethanol in a Waring blender and the mixture was run into a glass column fitted with a filter bed. Ethanol (*ca.* 1 l. per plant) was then allowed to percolate through the plant material slowly during 2 days. Evaporation of the ethanol left a residue which was vigorously shaken with water (50 ml.) until all the solid was dissolved or suspended. The solids were filtered off and dissolved in ethanol and Filtercel (*ca.* 0·1 g.) was added followed by water (50 ml.). After the ethanol had been evaporated, the mixture was filtered and the filtrate was combined with the first aqueous filtrate above. This solution was extracted thrice with light petroleum (b. p. 60—80°) and then with chloroform (8 × 200 ml.) which had previously been washed with 5% aqueous sodium carbonate. The chloroformic extracts were dried over anhydrous potassium carbonate and evaporated to a gum which was chromatographed on alumina in chloroform; the solvent had previously been washed as above and then dried over potassium carbonate. The fractions which crystallised readily on trituration with ethyl acetate were combined and recrystallised from this solvent to afford colchicine (usually 30—60 mg.). Further purification was achieved by dilution with pure non-radioactive colchicine and recrystallisation to constant activity.

Preparation and Reduction of Allocolchicine (II; R = CO_2Me).—The details in this section and in the ones below refer to the degradation of non-radioactive colchicine. Colchicine from the tracer experiments was degraded similarly and the products were identified by comparison with those obtained in the non-radioactive series.

Colchicine (404 mg.) was heated under reflux for 40 min. with methanolic sodium methoxide (3·2 ml. containing 1 g. of sodium in 10 ml. of methanol) and water (0·04 ml.). Water (45 ml.) was added to the reaction mixture and extraction with chloroform (3 × 50 ml.) removed a coloured impurity (5 mg.). After the aqueous solution had been acidified, it was extracted with chloroform (3 × 60 ml.) to afford allocolchiceine (II; R = CO_2H) m. p. 256° (from ethyl acetate) (lit.,[16] 254—255°). Samples for assay of radioactivity were prepared by percolation of a solution of allocolchiceine in 1 : 25 ethanol–chloroform through a short column of silica gel. The product from this was then sublimed at 260°/0·03 mm. to give material of m. p. 258° (Found: C, 65·2; H, 5·9; N, 3·75. Calc. for $C_{21}H_{23}NO_6$ C, 65·4; H, 6·0; N, 3·6%).

The foregoing material was methylated with ethereal diazomethane by Santavy's method [16] to give allocolchicine (II; R = CO_2Me) in almost quantitative yield, m. p. 250° (lit.,[16] 248°), $\nu_{max.}$ 1725 (CO_2Me), 1650 cm.$^{-1}$ (NH·CO), mass spectrometric mol. wt. 399 (Calc. for $C_{22}H_{25}NO_6$, 399).

Allocolchicine (0·8 g.) was slowly extracted from a Soxhlet thimble into a suspension of lithium aluminium hydride (4 g.) in boiling tetrahydrofuran (250 ml.). The mixture was heated under nitrogen for 8 hr., cooled, and treated with saturated aqueous sodium potassium tartrate (250 ml.). After the tetrahydrofuran had been evaporated, the suspension was extracted with chloroform (6 × 300 ml.) and the combined extracts were evaporated to *ca.* 100 ml. This solution was shaken twice with an excess of N-sulphuric acid and the chloroformic layer was dried and evaporated to yield the *acetamido-alcohol* (IV) as a gum (159 mg.). This crystallised from 1 : 4 chloroform–ether as prisms and was recrystallised from aqueous ethanol. A better yield was obtained by chromatographic purification of the crude product in chloroform on neutral alumina (Found: C, 64·6; H, 7·2; N, 3·55. $C_{21}H_{25}NO_5,H_2O$ requires C, 64·8; H, 7·0; N, 3·6%), $\nu_{max.}$ 3260 (OH), 1650 cm.$^{-1}$ (CO·NH), $\lambda_{max.}$ (in EtOH) 262 mμ (log ε 4·21).

The acidic solution above was basified with potassium carbonate and extracted with chloroform to afford the *ethylamino-alcohol* (III; R = OH) as a gum (598 mg.) which crystallised from ether as prisms, m. p. 126—127° (Found: C, 70·1; H, 7·3%; Equiv. 366. $C_{21}H_{27}NO_4$ requires C, 70·6; H, 7·6%; Equiv. 357), $\nu_{max.}$ 3260 cm.$^{-1}$ (OH), no carbonyl absorption, $\lambda_{max.}$ (in EtOH) 263 mμ (log ε 4·23).

Picric acid (0·4 g.) was added to a solution of this base (570 mg.) in ethanol (10 ml.), and,

[34] Cook, Johnstone, and Loudon, "The Alkaloids," ed. Manske and Holmes, Academic Press, New York, 1952, Vol. II, p. 266.

after the addition of water, the *base picrate* separated (733 mg.), m. p. 164° (decomp.) (Found: C, 55·3; H, 5·4; N, 9·65. $C_{27}H_{30}N_4O_{11}$ requires C, 55·3; H, 5·2; N, 9·55%).

When this reduction was run for 1 hr. and 2·75 hr., the yields of neutral material were 54 and 29%, respectively, and the remaining material was basic.

7-Ethylamino-1,2,3-trimethoxy-9-methyldibenzo[a,c]*cyclohepta-1,3-diene* (III; R = H).—Perchloric acid (0·83 ml.; 60%) and 10% palladised charcoal (0·3 g.) were added to a solution of the foregoing basic alcohol (564 mg.) in ethanol (100 ml.) and the suspension was shaken at room temperature and pressure; uptake (1·0 mol.) was complete in 0·5—2 hr. The catalyst was then filtered off, and the filtrate treated with water (50 ml.) and freed from ethanol by evaporation. Basification of the aqueous solution with potassium carbonate was followed by extraction with chloroform, to yield the base (III; R = H) as a gum (530 mg.). A solution of this in aqueous ethanol was treated with an excess of aqueous perchloric acid to give the *perchlorate*, m. p. 252° (Found: C, 56·9; H, 6·6. $C_{21}H_{28}ClNO_7$ requires C, 57·0; H, 6·4%). Microtitration of the recovered base gave an equiv. wt. of 353 (theory 341); $\nu_{max.}$ 3350 cm.$^{-1}$ (OH).

Hofmann Degradation of the Base (III; R = H).—An ethanolic solution (2 ml.) of the base (127 mg.) recovered from the foregoing perchlorate was treated with dimethyl sulphate (1 ml.) and N-sodium hydroxide (25 ml.) and the mixture was shaken at room temperature for 2 days. It was then adjusted to pH 8, treated with potassium iodide (10 g.), and freed from ethanol by evaporation. The *quaternary iodide* (V; X = I) separated (74 mg.) and more was obtained by extraction of the mother-liquors with chloroform, m. p. 195° (from chloroform–ether) (Found: C, 55·1; H, 6·5. $C_{23}H_{32}INO_3$ requires C, 55·5; H, 6·5%).

An alternative method involved heating a solution of the base (III; R = H) (350 mg.) and potassium carbonate (5 g.) in methanol (40 ml.), water (7·5 ml.), and methyl iodide (20 ml.) under reflux for 7 hr. Potassium iodide (25 g.) was added to the cooled solution and, after extraction thrice with ether, it was extracted thrice with chloroform. Evaporation of the latter extracts left a resin (464 mg.) which crystallised from chloroform–ether to afford the same quaternary iodide prepared above (m. p. and mixed m. p., infrared).

A solution of this iodide (412 mg.) in methanol (40 ml.) and water (30 ml.) was run through a column of Amberlite IRA-400 resin in the hydroxyl phase in apparatus which prevented the access of carbon dioxide. The percolate and aqueous washings were evaporated to dryness and the residue was heated at 10 mm. for 4 hr. at 100°. The products were separated into a water soluble fraction and an ether soluble fraction and the latter, as an ethereal solution, was shaken twice with an excess of 2N-sulphuric acid. Evaporation of the ether layer left a gum (144 mg.) which was purified by chromatography over alumina in benzene and the crystalline fractions (130 mg.) were recrystallised from aqueous ethanol to give 1,2,3-trimethoxy-9-methyldibenzo-[*a*,*c*]cyclohepta-1,3,5-triene (VII), m. p. 110° (Found: C, 77·15; H, 6·8. Calc. for $C_{19}H_{20}O_3$: C, 77·0; H, 6·8%), $\nu_{max.}$ 1610 cm.$^{-1}$ (C=C), $\lambda_{max.}$ (in EtOH) 239, 262 mμ (log ε 4·60, 414).

The acidic aqueous solution above was basified with potassium carbonate and extracted with chloroform, to yield a gum (63 mg.). This was treated with picric acid in aqueous ethanol and *7-dimethylamino-1,2,3-trimethoxy-9-methyldibenzo*[a,c]*cyclohepta-1,3-diene picrate* separated, m. p. 191—192° (decomp.) (Found: C, 57·3; H, 5·6; N, 9·6. $C_{27}H_{30}N_4O_{10}$ requires C, 56·8; H, 5·3; N, 9·8%).

The base recovered from the picrate as usual was methylated as above and the resultant quaternary iodide was subjected to Hofmann degradation as before. The neutral product was identical with that prepared earlier (m. p. and mixed m. p., infrared).

In trial experiments, the quaternary hydroxide (V; X = OH) (*ca.* 400 mg.) was evaporated to dryness with potassium hydroxide (0·4 g.) and the residue was heated at 120° for 2 hr. The neutral products were fractionated in benzene on alumina and the various fractions were combined on the basis of m. p. to give two portions which were recrystallised separately from aqueous ethanol. The crops had m. p. 85—87° and 111—112°. Part (8·9 mg.) of the former was hydrogenated in ethanol over 10% palladised charcoal in microapparatus (uptake 1·04 mol.) and the product was recovered as a gum. Similarly, part (9·6 mg.) of the latter was reduced (uptake 0·98 mol.) and the product was shown from its infrared spectrum to be identical with that obtained from the first reduction.

The Diol (VIII).—Osmium tetroxide (0·2 g.) was added to a solution of the cycloheptatriene (VII; 169 mg.) in ether (20 ml.) and after 6 days the ether was decanted from the precipitate. The latter was heated under reflux with water (15 ml.), methanol (15 ml.), and sodium sulphite (1·2 g.) for 2 hr., and the solids were filtered off. The concentrated filtrate was diluted with

water and extracted with ether; the extracted material crystallised from aqueous ethanol to afford the *diol* (VIII) as needles (132 mg.), m. p. 182° (Found: C, 69·0; H, 6·7. $C_{19}H_{22}O_5$ requires C, 69·1; H, 6·7%), $\lambda_{max.}$ (in EtOH) 261 mμ (log ε 4·28).

10-*Formyl*-2,3,4-*trimethoxy*-7-*methylphenanthrene* (IX; R = CHO) *and the Corresponding Acid* (IX; R = CO_2H).—A solution of the foregoing diol (322 mg.) in anhydrous benzene (60 ml.) was warmed to 40° and lead tetra-acetate (425 mg.) was added. After the solution had been heated to boiling, it was kept at room temperature for 5 hr. The solution was filtered, washed with water, and evaporated to a gum (327 mg.) which was boiled for 5 min. with aqueous methanol containing sodium carbonate (50 mg.). Evaporation of this solution left a residue which, in benzene (10 ml.), was chromatographed on alumina to afford the *aldehyde* (IX; R = CHO) as yellow needles (263 mg.), m. p. 95° (from aqueous ethanol) (Found: C, 73·4; H, 5·9. $C_{19}H_{18}O_4$ requires C, 73·5; H, 5·9%), $\nu_{max.}$ 2760, 1689 cm.$^{-1}$ (Ar.CHO), $\lambda_{max.}$ (in EtOH) 261, 332 mμ (log ε 4·71, 3·96).

A small yellow band (16 mg.), m. p. 171—173°, preceded the main one on the column and this is probably the 9-aldehyde, isomeric with structure (IX; R = CHO), $\nu_{max.}$ 1690 cm.$^{-1}$.

The major product (106 mg.), in acetone (6 ml.) and water (1·5 ml.), was treated at 60—70° with 2% aqueous potassium permanganate (7 ml.) during 30 min. After the mixture had been acidified and clarified by the addition of sodium pyrosulphite, it was extracted with ether and the combined extracts were shaken with an excess of aqueous sodium carbonate. Acidification of the alkaline solution and extraction with chloroform gave the acid (IX; R = CO_2H) as a crystalline solid. Purification was achieved by sublimation at 200° to give needles m. p. 205° (Found: C, 69·2; H, 5·8. Calc. for $C_{19}H_{18}O_5$: C, 69·9; H, 5·6%).

2,3,4-*Trimethoxy*-7-*methylphenanthrene* (IX; R = H).—The foregoing acid (80 mg.) was heated under reflux for 10 min. with quinoline (10 ml.) and copper chromite [35] (0·1 g.) while nitrogen, free from carbon dioxide, was passed through the apparatus and into saturated (at 20°) aqueous barium hydroxide which was heated to 70°. The precipitated barium carbonate was collected, washed with water, and dried at 100° for assay of radioactivity.

The quinoline solution was filtered, strongly acidified with hydrochloric acid, and extracted with chloroform. After the chloroformic solution had been washed with water, aqueous sodium carbonate, and again with water, it was evaporated and the residue (70 mg.) was fractionated on alumina in benzene. The main band crystallised from aqueous ethanol to yield 2,3,4-*trimethoxy*-7-*methylphenanthrene* as prisms (45 mg.), m. p. 94° (Found, in material dried at 65°: C, 76·6; H, 6·5. $C_{18}H_{18}O_3$ requires C, 76·6; H, 6·4%), $\lambda_{max.}$ (in EtOH) 258, 282, 302 mμ (log ε 4·97, 4·25, 4·04).

Benzene-1,2,4-*tricarboxylic Acid* (*Trimellitic Acid*).—2,4-Dimethylacetophenone (5 g.) in boiling water was stirred as aqueous potassium permanganate (5% w/v, 600 ml.) was added during 4 hr. Solid potassium permanganate (5 g.) was then added portionwise during the next 20 hr. and the cooled solution was then treated with hydrogen peroxide to remove the excess of permanganate. The mixture was filtered, the filtrate was evaporated to *ca.* 100 ml., extracted with chloroform, acidified, and extracted continuously with ether for 8 hr. Evaporation of the ether left a solid (6 g.). Part (1·5 g.) was heated in a sublimation apparatus at *ca.* 11 mm. and the fraction volatilising at 195—200° was collected (0·75 g.). A portion (42 mg.) was resublimed and crystallised from ether–benzene, to give trimellitic anhydride (37 mg.) m. p. 163—165° (lit.,[16] m. p. 163°) (Found: C, 56·25; H, 2·2. Calc. for $C_9H_4O_5$: C, 56·25; H, 2·1%). This material was used to control the experiments on allocolchiceine, below, which gave very variable yields. The following two procedures were the best of many tested.

Allocolchiceine (0·3 g.) in aqueous nitric acid (12% w/v; 3 ml.) and acetic acid (3 ml.) was heated on a steam-bath for 5 min. The solution was then poured into N-sodium hydroxide (50 ml.) and saturated (at 20°) aqueous potassium permanganate (30 ml.) was added. After the mixture had been boiled under reflux for 1·5 hr., it was acidified and boiled for a further 1 hr. with the permanganate solution (20 ml.). Sodium pyrosulphite was added to the cooled mixture and the clear solution was continuously extracted with ether, to yield an acidic gum. This was purified by sublimation, as above, to give trimellitic anhydride, identical with the standard sample (m. p. and mixed m. p.). The yield varied between 3 and 30%.

Allocolchiceine (0·3 g.) was heated with concentrated hydrochloric acid (3 ml.) at 170° in a sealed tube for 1·75 hr. The resultant mixture was poured into water (300 ml.) containing

[35] Adkins and Connor, *J. Amer. Chem. Soc.*, 1931, **53**, 1091.

potassium hydroxide (20 g.) and potassium ferricyanide (75 g.) and the solution was stirred on a steam-bath for 12 hr., when a second addition of potassium hydroxide (13 g.) and potassium ferricyanide (75 g.) was made. The same amounts were added after 36 hr. After 60 hr., the cooled solution was filtered, and the filtrate was acidified with sulphuric acid and then extracted continuously with ether to give a gum (0·2 g.). A solution of this in boiling water (10 ml.) was treated portionwise with potassium permanganate (an excess) during 1 hr. The acidified mixture was then worked up for trimellitic anhydride as in the foregoing method; the yields in two runs were 30 and 5%.

Oxidation of Colchicine.—(a) *By ozone.* Ozonised oxygen (*ca.* 4% O_3) was passed for 5·5 hr. through a solution of colchicine (1·79 g.) in formic acid (30 ml.). Formic acid (20 ml.) and hydrogen peroxide (18 ml.; 100 vol.) were then added and the solution was heated at 60° for 1·5 hr. before being extracted with chloroform (4 × 50 ml.). The residue from evaporation of the aqueous layer was dissolved in water (20 ml.) and ozonised oxygen was passed through the solution for 0·5 hr. Further portions of formic acid and hydrogen peroxide (18 ml.) were then added and, after this solution had been heated at 60° for 1 hr., it was evaporated to dryness. Saturated barium hydroxide solution was added until an alkaline solution was obtained which yielded no further precipitate on the addition of barium hydroxide. The solids were filtered off, and the filtrate, concentrated to 10 ml., was acidified with sulphuric acid and extracted continuously with ether. After 2 hr., the ethereal extract was evaporated and the residue was recrystallised thrice from acetone to give succinic acid (22·5 mg.). Part was neutralised with 0·074N-lithium hydroxide (carbonate-free; 2·39 ml.), and the solution was concentrated to 0·5 ml. (with exclusion of carbon dioxide) before *p*-bromophenacyl bromide (52 mg.) in hot ethanol (20 ml.) was added. The solution was boiled almost to dryness during 50 min., and from the cold solution the crystals were collected and recrystallised from ethanol, to give di-*p*-bromophenacyl succinate (12 mg.), m. p. 212—213° (lit.,[36] 212—213°).

The ether extraction (above) was continued, and after 24 hr. the extract was evaporated and the residue combined with the material in the mother-liquors from the crystallisation of succinic acid. This mixture was fractionated by chromatography on preparative thin-layer plates in benzene–methanol–acetic acid (45 : 8 : 4 by vol.) using authentic *N*-acetyl-L-glutamic acid as a control. The material eluted with methanol from the appropriate section of the plates was dissolved in acetone, the solution was filtered, and the residue from evaporation of the filtrate was crystallised from water to give buff crystals (17 mg.). These were recrystallised from acetone, ethanol, and finally water, to give pure *N*-acetyl-L-glutamic acid, m. p. 191—192° (lit.,[37] 193—194°), identified by direct comparison with authentic material (Found: C, 44·3; H, 5·8; N, 7·4. Calc. for $C_{20}H_{23}NO_4$: C, 44·4; H, 5·9; N, 7·4%).

The material in the mother liquors from the crystallisation of *N*-acetyl-L-glutamic acid was recovered and heated at 110° in an evacuated sealed tube with 6N-hydrochloric acid (0·7 ml.) for 12 hr. Steam-distillation gave aqueous acetic acid (70 ml.) which was neutralised with 0·074N-lithium hydroxide (2·7 ml.) and evaporated to dryness. A solution of *p*-bromophenacyl bromide (59 mg.) in ethanol (30 ml.) was added to the residue and this solution was boiled to dryness over 0·5 hr. The benzene-soluble portion of the residue was chromatographed on silicic acid (2·5 g.) and the appropriate fractions (control by thin-layer chromatography) were combined and evaporated. Crystallisation of the residue from ethanol gave *p*-bromophenacyl acetate (11 mg.), m. p. 84—85°.

The solution in the steam-distillation flask was basified with sodium hydroxide and then shaken vigorously with an excess of phenyl isocyanate at 50° for 30 min. Diphenylurea was filtered off and the filtrate was extracted thrice with ether, acidified, and extracted again with with ether (4 × 70 ml.). The latter extract was dried and evaporated, and the residue crystallised from ethyl acetate, to give the *N*-phenylurea of L-glutamic acid (6·4 mg.), m. p. 105—106° (Found: C, 54·3; H, 5·5; N, 10·5. Calc. for $C_{12}H_{14}N_2O_5$: C, 54·1; H, 5·3; N, 10·5%). This material was identical with an authentic sample prepared from L-glutamic acid and phenyl isocyanate, $[\alpha]_D^{27}$ +9·5° (*c* 0·6 in acetone).

(b) *By chromic acid.* Chromium trioxide (4·06 g.) and concentrated sulphuric acid (3 ml.) were added to a solution of colchicine (445 mg.) in water (19 ml.), and the mixture was heated under reflux for 1 hr. and kept at room temperature for 2·5 hr. Sufficient methanol was added

[36] Schmid and Yeowell, *Experientia*, 1964, **20**, 250.
[37] Nicolet, *J. Amer. Chem. Soc.*, 1930, **52**, 1192.

to destroy the excess of oxidising agent, and the solution (concentrated to 8 ml.) was extracted continuously with ether for 18 hr. The extracted material crystallised from acetone to give succinic acid (21·5 mg.) which was sublimed at 70—80°/10^{-4} mm. Part was converted into the di-*p*-bromophenacyl ester as above, m. p. 212—213°. A further portion of the succinic acid (1·224 mg.) was heated with an excess of polyphosphoric acid [38] and sodium azide (6 mg.) at 110—114° in a high vacuum for 4 hr. in equipment which allowed the generated carbon dioxide (32%) to be trapped and measured manometrically. The gas was absorbed in tetramethylammonium hydroxide (*ca.* 30 mg.) and triethyleneglycol (1 drop) for determination of radioactivity by solution scintillation counting.

7-Acetamido-1,2,3-trimethoxydibenzo[a,c]*cyclohepta-1,3-diene* (II; R = H).—Allocolchiceine (422 mg.) was heated under reflux with copper chromite in quinoline (12 ml.) for 1·5 hr. while a stream of nitrogen swept the evolved carbon dioxide into barium hydroxide solution, as above. The barium carbonate was collected (164 mg.).

After the reaction solution had been evaporated to 3 ml., it was diluted with benzene (180 ml.) and shaken with hydrochloric acid (3 × 80 ml.). After the benzene layer had been washed with N-sodium hydroxide, it was dried and evaporated, to give a gum (285 mg.) which was chromatographed on alumina (5 g.) in ethyl acetate. The crystalline fractions (245 mg.) were sublimed at 170°/5 × 10^{-5} mm. and recrystallised from ethanol, to give the *amide* (210 mg.), m. p. 185—186° (Found: C, 70·4; H, 6·6%; *M*, 341 (mass spectrometry). $C_{20}H_{23}NO_4$ requires C, 70·4; H, 6·8%; *M*, 341).

Oxidation of the Cycloheptadiene (II; R = H).—Potassium permanganate (1 g.) and sodium hydroxide (360 mg.) were added to a suspension of the cycloheptadiene (198 mg.) in water (70 ml.), and the mixture was heated under reflux for 18 hr. More permanganate (240 mg.) was added, and after the solution had been heated for a further 75 min. it was acidified with concentrated sulphuric acid and heated at 50° for 1 hr. with a third portion (250 mg.) of permanganate. The basified solution was filtered, evaporated to 20 ml., and extracted with benzene and then with ether before being acidified and re-extracted with ether (6 × 60 ml.). The last extracts afforded an oil (144 mg.) from which was sublimed crude phthalic anhydride (49 mg.) at 130°/1·5 mm. Part (16 mg.) was treated with aniline (25 mg.) and acetic acid (0·45 ml.), and after the mixture had been heated under reflux for 2·5 hr. it was worked up as usual for neutral material. This was sublimed at 110—130°/1·1 mm., to give crystals (7·6 mg.); recrystallisation twice from ethanol yielded *N*-phenylphthalimide (4·3 mg.), m. p. 210—211°, which was identical with an authentic sample (Found: C, 75·2; H, 3·9; N, 6·4. Calc. for $C_{14}H_9NO_2$: C, 75·3; H, 4·1; N, 6·3%).

The remaining crude phthalic anhydride was heated at 120° for 30 min. with concentrated sulphuric acid and sodium azide (43 mg.). After the cooled solution had been adjusted to pH 5 with sodium hydroxide, it was extracted with ether (6 × 12 ml.) to yield crude anthranilic acid (21 mg.). A solution of this in 2N-sodium hydroxide (1·5 ml.) was shaken with benzoyl chloride (125 mg.) at 50° for 10 min., and after being kept at room temperature for 30 min., the solution was acidified. The solid was collected and recrystallised from water and then from ethanol, to give *N*-benzoylanthranilic acid (7·9 mg.), m. p. 182° (Found: C, 69·4; H, 4·7; N, 5·9. Calc. for $C_{14}H_{11}NO_3$: C, 69·7; H, 4·6; N, 5·8%).

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[38] Arigoni, personal communication. We thank Professor D. Arigoni for information concerning this method.