Biosynthesis. Part XXI.[1,2] Investigations on the Biosynthesis of Stylopine in *Chelidonium majus*

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Methods are described for the degradation of stylopine (2,3:9,10-bismethylenedioxyberbine) (6) which allow radioassay of various parts of the stylopine molecule. Samples of stylopine isolated from *C. majus* plants fed with a variety of multiply labelled precursors have been degraded by these methods, and these and other results show that (*a*) (+)-(*S*)-reticuline (4) is the precursor of (−)-(*S*)-stylopine (6); (*b*) C-1 and C-9 of reticuline are unaffected during its conversion into stylopine; (*c*) C-8 of stylopine is formed by oxidative cyclisation involving the *N*-methyl group of reticuline and this step involves an appreciable isotope effect; and (*d*) a small pool of scoulerine [as (5)] is present in *C. majus* plants, and the conversion of (−)-(*S*)-scoulerine (5) into (−)-(*S*)-stylopine (6) is demonstrated to occur *in vivo* without loss of tritium from the chiral centre. Some experiments on the rearrangement of *N*-oxides *in vitro* are described.

We have long been attracted by the possibility [3] that several important classes of alkaloids are formed in nature by modification of the tetrahydroprotoberberine (berbine) skeleton [*e.g.* (5)]. Experimental proof has already been published [4] that narcotine (1), a representative of the phthalideisoquinoline class, is formed in this way [(5) ⟶ (1)]. Our interest in the biosynthesis of chelidonine (2) led us to study the tetrahydroprotoberberine (6), which in principle (see following paper) ought to be the precursor of chelidonine; the base (6) is called stylopine and it occurs with chelidonine in the greater celandine (*Chelidonium majus*).

Berberine (3) was already known to be built from two C_6–C_2 units derivable from tyrosine.[5] Also it had been discovered that the ' berberine bridge ' [C-8 of structure (3)] arises [6,7] by oxidative cyclisation involving the *N*-methyl group of a 1-benzylisoquinoline. The Imperial College group [6] used doubly labelled reticuline (9) with *Hydrastis canadensis* plants and we used [N-*methyl*-^{14}C, 3-^{14}C]laudanosoline (10) for incorporation into berberine in *Berberis japonica* plants.[7] Details of our work on the berberine bridge are given in the Experimental section.

We decided for the study of stylopine (6) to move directly into experiments on the late stages of biosynthesis as follows.

Experiments with Multiply Labelled Reticuline.—The synthesis of (*RS*)-[1-^{3}H, 3-^{14}C, N-*methyl*-^{14}C, 4′-O-*methyl*-^{14}C]reticuline (12) was described earlier [8] and this product was resolved into *R*- and *S*-forms at that time.[8] The proportion of the total activity at each of the illustrated ^{14}C-labelled positions has since been determined by degradation [9] of the radioactive *RS*-material as in Scheme 1. Radioassay of the three final degradation products provided the results and proportional values in Table 1.

The multiply labelled *S*- and *R*-reticulines (4) and (7) were administered to *Chelidonium majus* plants, which after 5—7 days were worked for alkaloids. The isolated stylopine was found to be a partial racemate containing an excess of the (−)-(14*S*)-form (6). Experiments 1 and 2 in Table 2 show that (+)-(*S*)-reticuline (4) is a much

TABLE 1

Degradation of labelled (*RS*)-reticuline (12)

	6-Ethylveratric acid (15)	Veratric acid (13)	Trimethylamine picrate
Specific activity (disint. s^{-1} $mmol^{-1}$)	$6{\cdot}8 \times 10^4$	$1{\cdot}2 \times 10^4$	$9{\cdot}5 \times 10^3$
Relative molar activity	0·76	0·13	0·11
Symbol	■	●	▲

TABLE 2

Tracer experiments on *Chelidonium majus* plants

Expt. no.	Precursor	Incorpn. (%) into stylopine [a] (and ^{3}H : ^{14}C ratio)
1 [b]	(+)-(*S*)-[1-^{3}H, 3-^{14}C, N-*methyl*-^{14}C, 4′-O-*methyl*-^{14}C]Reticuline (4)	0·06
2 [b]	(−)-(*R*)-[1-^{3}H, 3-^{14}C, N-*methyl*-^{14}C, 4′-O-*methyl*-^{14}C]Reticuline (7)	0·013
3	(±)-(*RS*)-[1-^{3}H, 3-^{14}C, N-*methyl*-^{14}C, 4′-O-*methyl*-^{14}C]Reticuline	0·05
4 [c]	(+)-(*S*)-[3-^{14}C, N-*methyl*-^{3}H]Reticuline (^{3}H : ^{14}C ratio 11·1 : 1)	0·50 (11·9 : 1)
5 [c]	(−)-(*R*)-[3-^{14}C, N-*methyl*-^{3}H]Reticuline (^{3}H : ^{14}C ratio 11·1 : 1)	0·028 (9·3 : 1) [f]
6 [d]	(+)-(*S*)-[3-^{14}C, 9-^{3}H]Reticuline (^{3}H : ^{14}C ratio 12·0 : 1)	0·53 (11·5 : 1)
7 [d]	(−)-(*R*)-[3-^{14}C, 9-^{3}H]Reticuline (^{3}H : ^{14}C ratio 12·0 : 1)	0·018 (11·8 : 1)
8	(−)-(*S*)-[1,12-^{3}H]Scoulerine (5)	0·40 [g]
9 [e]	(−)-(*S*)-[6-^{14}C, 14-^{3}H]Scoulerine (5) (^{3}H : ^{14}C ratio 5·07 : 1)	0·28 (5·58 : 1)
10 [e]	(+)-(*R*)-[6-^{14}C, 14-^{3}H]Scoulerine (8)	0·013

[a] Calc. on wt. of first crop of stylopine base which is a partial racemate containing an excess of (−)-form. [b–d] Parallel experiments. [e] Parallel experiments carried out by Dr. R. Southgate. [f] Reduced accuracy because of low activity. [g] Also 0·2% into coptisine chloride.

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‡ Dedicated to Professor F. Sǎntavý, Olomouc, Czechoslovakia, on the occasion of his sixtieth birthday.

[1] Part XX, A. R. Battersby, P. Böhler, M. H. G. Munro, and R. Ramage, *J.C.S. Perkin I*, 1974, 1399.

[2] Preliminary reports of the early part of this work, A. R. Battersby, R. J. Francis, E. A. Ruveda, and J. Staunton, *Chem. Comm.*, 1965, 89; A. R. Battersby, R. J. Francis, M. Hirst, R. Southgate and J. Staunton, *ibid.*, 1967, 602.

[3] R. Robinson, ' The Structural Relations of Natural Products,' Clarendon Press, Oxford, 1955.

[4] A. R. Battersby, M. Hirst, D. J. McCaldin, R. Southgate, and J. Staunton, *J. Chem. Soc.* (*C*), 1968, 2163.

[5] J. R. Gear and I. D. Spenser, *Canad. J. Chem.*, 1963, **41**, 783; I. Monković and I. D. Spenser, *Proc. Chem. Soc.*, 1964, 223.

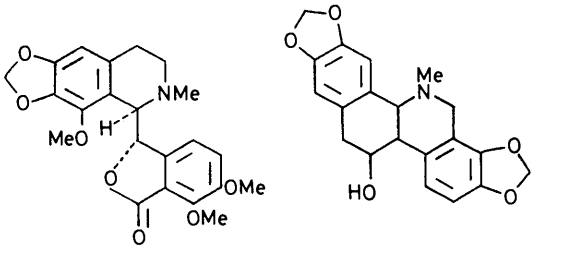
[6] D. H. R. Barton, R. H. Hesse, and G. W. Kirby, *Proc. Chem. Soc.*, 1969, 267; *J. Chem. Soc.*, 1965, 6379.

[7] A. R. Battersby, R. J. Francis, M. Hirst, and J. Staunton, *Proc. Chem. Soc.*, 1963, 268.

[8] A. R. Battersby, D. M. Foulkes, and R. Binks, *J. Chem. Soc.*, 1965, 3323.

[9] A. R. Battersby and B. J. T. Harper, *J. Chem. Soc.*, 1962, 691.

better precursor of stylopine than is (—)-(*R*)-reticuline (7); even greater discrimination in favour of (*S*)-reticuline was observed in later work (*e.g.* experiments 6 and 7, Table 2).



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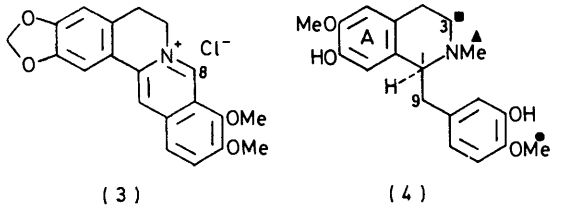
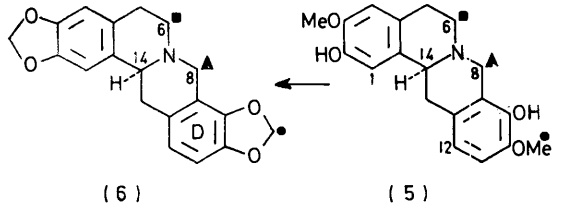
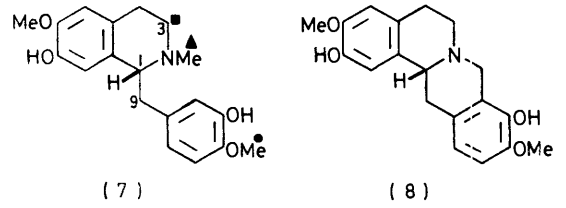


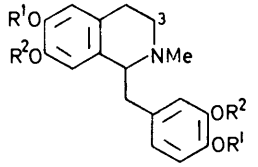
(3) (4)



(6) (5)

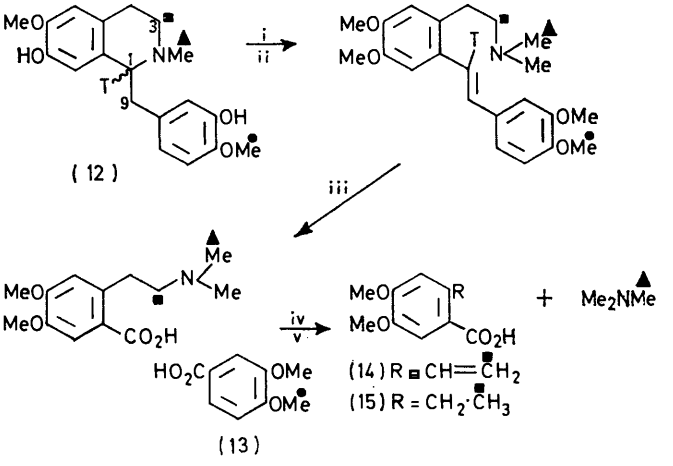


(7) (8)



(9) R^1 = Me R^2 = H
(10) R^1 = R^2 = H
(11) R^1 = R^2 = Me

Degradation of the labelled stylopine from experiment 1 was first attempted by Hofmann degradation (Scheme 2) to the methine (16). This was cleaved with osmium tetraoxide–periodate and the molar specific activity of the resultant formaldehyde (dimedone derivative) corresponded to 52% of that of the original stylopine. The expected value (see ■, Table 1) was 76% and we suspected dilution with unlabelled formaldehyde arising from oxidation elsewhere in the molecule; the *N*-methyl [1] or methylenedioxy-groups are likely sources. Support for this interpretation was gained by catalytic reduction of the methine to the corresponding *C*-ethyl derivative (17), which was treated with osmium tetraoxide–periodate and worked up as before with addition of radioactive formaldehyde dimedone derivative of known quantity and specific activity. This reverse dilution analysis showed that 0·18 mol. equiv. of formaldehyde had been formed from the *C*-ethyl derivative (17).



(12) (13) (14) R = CH=CH$_2$ (15) R = CH$_2$·CH$_3$

SCHEME 1 Reagents: i, MeI; ii, hot KOH; iii, $KMnO_4$; iv, Me_2SO_4 then hot KOH; v, H_2, Pt.

Accordingly two separate degradations were carried out (Scheme 2). Stylopine from experiment 1 was oxidised with iodine to coptisine iodide (18; X = I), which with phenylmagnesium bromide yielded phenyldihydrocoptisine (20); part of the latter was reduced with borohydride to phenyltetrahydrocoptisine (21) for radioassay. Oxidation of the dihydro-derivative (20) with permanganate yielded benzoic acid. A parallel run was carried out on stylopine from experiment 3. The second sequence was based upon that developed by Gear and Spenser [10] for degradation of berberine. Stylopine from experiment 3 was converted as before into coptisine iodide and then *via* the sequence (18) ⟶ (22) ⟶ (23) ⟶ (24) into hydrastinine (25) and the aldehyde (26). The radio-assays are collected in Table 3 for all three degradations.

A brief study was made of the location of tritium in the (*RS*)-stylopine from experiment 1, Table 2; this sample was oxidised as before to coptisine iodide (18; X = I), which was converted by ion-exchange into the chloride before purification. The chloride (18; X = Cl) was then reduced with borohydride to (*RS*)-stylopine; this product carried less than 7% of the starting ^3H activity in accord with its being located originally at C-14. In another experiment, (*RS*)-[3-^{14}C, 1-^{3}H]reticuline [8] [as (12); ^3H : ^{14}C ratio 1·47 : 1] was administered to *C.*

[10] J. R. Gear and I. D. Spenser, *Canad. J. Chem.*, 1963, **41**, 783.

TABLE 3

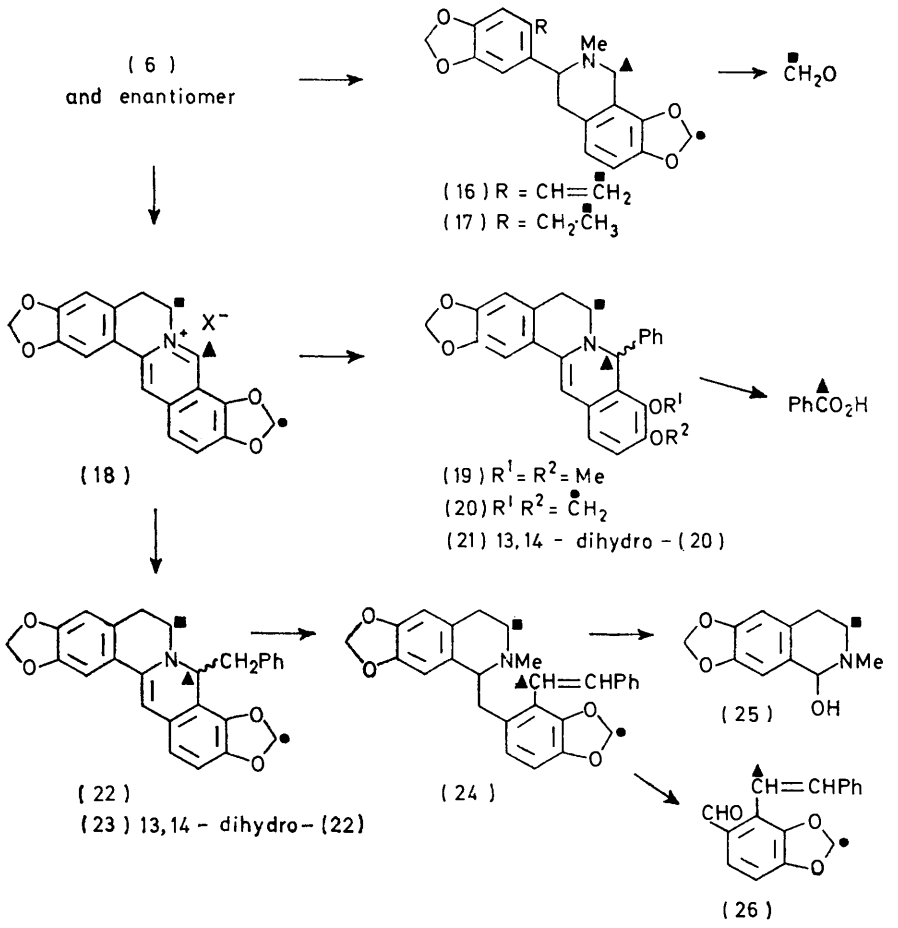
Degradation of labelled stylopine [as (6)]

	Specific activity (disint. per mmol) [Relative molar activity]		Expected molar values
	From expt. 1, Table 2	From expt. 3, Table 2	
Stylopine [(6) + enantiomer]	$7\cdot3 \times 10^4$ [1·00]	$6\cdot1 \times 10^4$ [1·00]	1·00
Phenyltetrahydrocoptisine (21)	$7\cdot3 \times 10^4$ [1·00]	$6\cdot1 \times 10^4$ [1·00]	1·00
Benzoic acid	$8\cdot3 \times 10^3$ [0·11]	$5\cdot9 \times 10^3$ [0·10]	0·11
Benzyltetrahydrocoptisine (23)		$6\cdot4 \times 10^4$ [1·05]	1·00
Methine (24)		$5\cdot9 \times 10^4$ [0·97]	1·00
Hydrastinine (25)		$4\cdot5 \times 10^4$ [0·74]	0·76
Aldehyde (26)		$1\cdot4 \times 10^4$ [0·23]	0·24

majus plants and the isolated stylopine was separated by fractional crystallisation from benzene–methanol into (*RS*)-stylopine [as (6)] and (−)-(*S*)-stylopine (6), $[\alpha]_D^{23}$ −310° (*c* 0·3 in $CHCl_3$). Both samples retained within experimental error all the original tritium (104 and 94%, respectively). Moreover, the specific activity of (−)-(*S*)-stylopine was 1·6 times that of (*RS*)-stylopine. Further work would be needed for complete understanding of these results but it is clear that (−)-(*S*)-stylopine (6) is on the main pathway.

The results so far show that (*a*) (+) (*S*)-reticuline (4) is the precursor of stylopine and that it is incorporated at least largely * intact; (*b*) C-8 of stylopine is formed by oxidative cyclisation involving the *N*-methyl group of (+)-(*S*)-reticuline (*cf.* berberine above); and (*c*) the methylenedioxy-group on ring D of stylopine arises from the *ortho*-hydroxy-methoxy-system of reticuline as in other cases (*e.g.* refs. 6 and 11).

When (+)-(*S*) and (−)-(*R*)-[N-*methyl*-^{3}H, 3-^{14}C]-reticulines [as (4) and (7), respectively] were administered to *C. majus* plants, the $^3H : {}^{14}C$ ratio in the isolated stylopine was found to be slightly higher than that in the precursor (experiments 4 and 5, Table 2). Small increases in $^3H : {}^{14}C$ ratio of *ca.* 10% have been observed in several other cases (*e.g.* see below) and are thought to be due to preferential removal of ^{14}C-labelled species



SCHEME 2

along other pathways (*i.e.* ' protection ' by ^{3}H labelling). However, we considered that the operation of a tritium

* There is no evidence that the carbon of the *O*-methyl group on ring A survives unchanged.

[11] A. R. Battersby and M. Hirst, *Tetrahedron Letters*, 1965, 669.

isotope effect would best account for the large difference between the observed result and the value of 66% retention expected statistically in the absence of such an effect. A related case was studied *in vitro*. Rearrangement of the *N*-oxide of (*RS*)-[N-*methyl*-^{3}H,^{14}C]laudanosine * (11) with iron(III) nitrate and oxalic acid [12] produced formaldehyde with ^{3}H : ^{14}C ratio indistinguishable from that of the original *N*-methyl group. Approximately 97% of the tritium would be retained if $K_H/K_T = 9$, so an appreciable isotope effect is clearly operative in this rearrangement. A number of simple amines and *N*-oxides prepared for preliminary experiments are described in the Experimental section.

The following paper will include some data relevant to the biosynthesis of stylopine and will also bring out the importance of C-9 in reticuline (4) for research on chelidonine (2). This centre has been studied here for the biosynthesis of stylopine by incorporation experiments with (+)-(*S*)- and (−)-(*R*)-[3-^{14}C, 9-^{3}H]reticulines.[4] The results in Table 2 (experiments 6 and 7) show that within experimental error no loss of tritium occurs during the biosynthetic conversion of reticuline into stylopine.

Experiments with Tetrahydroprotoberberines (Scoulerine).—The product of oxidative ring closure at the *N*-methyl group of (+)-(*S*)-reticuline (4) considered above should be (−)-(*S*)-scoulerine (5); the importance of this base has been emphasised by earlier studies.[4,13] Its role in the biosynthesis of stylopine was defined by preparing (−)-(*S*)-[1,12-^{3}H]scoulerine (5) by base-catalysed exchange with tritiated water. Solubility problems were overcome by using the tetramethylammonium salt of scoulerine. In addition, we synthesised (−)-(*S*)- and (+)-(*R*)-[6-^{14}C, 14-^{3}H]scoulerine [as (5) and (8), respectively] by the route developed earlier [13] starting from (−)-(*S*)-[1-^{3}H, 3-^{14}C]nor-reticuline [4] [as (4; NH in place of NMe)] and its enantiomer. Experiments 8 and 9, Table 2, show that (*a*) (−)-(*S*)-scoulerine is an effective precursor of (−)-(*S*)-stylopine; and (*b*) tritium is not lost from the chiral centre in the biochemical conversion. Indeed, a small but significant rise (10%) in ^{3}H : ^{14}C ratio is observed. We consider that this indicates a slight preferential removal of ^{14}C-labelled material along pathways in which the first step is oxidative attack at the C–H(T) residue at C-14 of (−)-(*S*)-scoulerine (5).

The above evidence can be strengthened if scoulerine is demonstrated to be present in *C. majus* plants; it had not been isolated previously from this source. Accordingly, radioinactive (*RS*)-scoulerine [(5) and (8)] was added to the total alkaloidal extract from experiment 4, Table 2, and was then reisolated and rigorously purified. Its activity corresponded to an incorporation of 3×10^{-4}%, a low but real value as shown by *O*-methylation of the isolated scoulerine to yield the di-*O*-methyl derivative [(*RS*)-tetrahydropalmatine]. This was further fractionated as its crystalline hydrochloride without change in radioactivity. A small pool of scoulerine is thus present in *C. majus* plants, a result in agreement with its rapid turnover into the alkaloids further along the biosynthetic route (*e.g.* stylopine and chelidonine).

The pathway (+)-(*S*)-reticuline (4) ⟶ (−)-(*S*)-scoulerine (5) ⟶ (−)-(*S*)-stylopine (6) is thus defined and our results provide information about stereochemical relations and oxidation levels at key centres which any mechanistic proposals must accommodate.

EXPERIMENTAL

For most general chemical and radiochemical directions, see ref. 14. In addition, for preparative layer chromatography (p.l.c.) 20 × 20 × 0·1 cm thick plates were made up with silica GF_{254}, and the required zones were eluted with 1 : 9 methanol–ether, unless otherwise stated. Aqueous solutions were extracted twice or thrice with equal volumes of solvents and back-washed once, and organic solutions were dried over sodium sulphate or magnesium sulphate and evaporated at about 40 °C under reduced pressure. Radioactive samples were counted in 7 ml of scintillator solution, and the efficiencies were determined by internal standardisation. Ether, hexane, and benzene were dried with sodium wire and dimethylformamide with molecular sieves. Pyridine was dried over barium oxide, then distilled, and dioxan and tetrahydrofuran were distilled from lithium aluminium hydride.

Plant Cultivation and Administration of Precursors.—*Berberis japonica* and *Chelidonium majus* plants were grown in the open. Young stems of the former (*ca.* 25 cm long) were dipped into an aqueous solution of the precursor hydrochloride held in small test tubes. Distilled water was added to the tubes from time to time as uptake demanded. When at least four times the original volume of solution had been absorbed, the stems were transferred to beakers of distilled water and allowed to metabolise for 5 days.

Precursors were introduced during the period late June to mid-August into *C. majus* either by injection of aqueous solutions of salts (usually hydrochlorides) directly into the hollow stems of whole plants or by cutting stems off (*ca.* 40 cm long) and following the procedure above. The second method is easier and generally gives higher incorporations. The amount of precursor which was not taken up from the tubes was determined by decolourising the combined aqueous residues with borohydride and a sample was then assayed for radioactivity. Normally >95% of the precursor was taken up by the plant material. In all cases, the maximum quantity of precursor introduced into each plant or shoot was 1 mg (average 0·5 mg). The initial concentration in the solutions of precursors was *ca.* 2 mg ml^{-1}. Harvested plants were stored at −30 °C until they were extracted.

Extraction and Separation of Alkaloids.—(a) *Whole plants.* Six *C. majus* plants were cut into pieces and macerated with methanol or ethanol in a Waring blender. The slurry was packed into a glass column and percolated with methanol or ethanol until the extracts were almost colourless (usually 5—10 l). Evaporation of the alcoholic solution left a

* The sample also carried a ^{14}C label at C-3, but for simplicity the data are reported only with respect to the doubly labelled *N*-methyl group.

[11] K. W. Bentley and A. W. Murray, *J. Chem. Soc.*, 1963, 2497; C. C. Sweeley and E. C. Horning, *J. Amer. Chem. Soc.*, 1957, **76**, 2620 and earlier papers.

[13] A. R. Battersby, R. Southgate, J. Staunton, and M. Hirst, *J. Chem. Soc.* (*C*), 1966, 1052.

[14] P. G. Strange, J. Staunton, H. R. Wiltshire, A. R. Battersby, K. R. Hanson, and E. A. Havir, *J.C.S. Perkin I*, 1972, 2364.

residue which was partitioned between N-hydrochloric acid (400 ml) and ether (4 × 200 ml) and the organic phases were back-washed with water (50 ml). The combined aqueous solution was extracted with chloroform (5 × 100 ml) and the combined organic layers were washed with water, then thoroughly shaken with an excess of saturated sodium carbonate solution and evaporated (extract A).

After the combined aqueous solution had been basified by mixing cautiously with the foregoing carbonate solution, it was extracted with chloroform (5 × 200 ml), and the combined chloroformic solution was washed with brine (50 ml) and evaporated to give extract B.

The foregoing aqueous layer was acidified with concentrated hydrochloric acid and extracted continuously with chloroform until 1 h of extraction yielded negligible material (12—30 h). Evaporation left extract C.

Extract A generally crystallised to give stylopine, which on recrystallisation from chloroform–methanol gave first the partial racemate with an excess of the (−)-(*S*)-form. Several recrystallisations, or (faster) crystallisation from benzene–methanol, afforded (*RS*)-stylopine (typically 40 mg), m.p. 220—221° (lit.,[15] 220—221°). The mother liquor from the first crystallisation was evaporated to small volume, acidified with a few drops of concentrated hydrochloric acid, and then diluted with ether to precipitate crude sanguinarine chloride (see following paper).

Extract B in the minimum volume of 1 : 1 benzene–chloroform was run onto a column of alumina (Spence, activity I; 250 g) and elution was continued with 3 : 7 benzene–chloroform. Sanguinarine and relatives were eluted first as their pseudobases, followed by chelidonine, which was crystallised as the base (typically 500 mg) from ethanol (m.p. 135—136°) and as the hydrochloride from aqueous methanol or benzene–methanol [m.p. 280—286° (decomp.)]. Further elution of the column gave protopine (50 mg), m.p. 209—210° (from methanol–chloroform) (lit.,[16] 207, 211°).

Extract C in methanol (10 ml) was treated with a few drops of concentrated hydrochloric acid and the solution was evaporated until the coptisine chloride crystallised. Recrystallisation from methanol–propan-2-ol (+ 1 drop hydrochloric acid) gave the pure salt (80 mg), m.p. 335° (decomp.).

The crude sanguinarine chloride from extract A and similar fractions containing this coloured alkaloid and its relatives from extract B were combined and fractionated on alumina (Spence, activity I; 100 g). The column was made up in 1 : 19 ethanol–chloroform (300 ml) saturated with hydrogen chloride and was washed with chloroform (300 ml), also saturated with hydrogen chloride. A solution of the alkaloids in methanol–benzene was mixed with alumina (10 g) and evaporated to dryness and the residue as a slurry in 1 : 1 benzene–chloroform was added to the top of the prepared column. Elution with a saturated solution of hydrogen chloride in chloroform gave, in well separated fractions, sanguinarine chloride (60 mg), m.p. 277—280° (decomp.) (from methanol–ether), and chelerythrine chloride (10 mg), m.p. 188—190° (decomp.) (from methanol–ether).

(b) *Cut stems*. The small quantity of alkaloid being handled required modification of the above methods. Radioinactive (*RS*)-stylopine, chelidonine, protopine, or allocryptopine (50 mg each) was added to the initial methanolic extract of the plant material and the first partition was between N-hydrochloric acid (100 ml) and ethyl acetate (100 ml). Extracts A—C were then obtained as above. The (*RS*)-stylopine from extract A was purified by p.l.c. with 3 : 7 hexane–ether (R_F 0·6) before final crystallisation; canadine showed R_F 0·5 in this system.

Purification of extract B on alumina (20 g; grade III) in 1 : 1 chloroform–benzene gave chelidonine, protopine, and allocryptopine in admixture; they were separated by p.l.c. with ether. Chelidonine was best handled as its *O*-acetyl derivative, prepared by shaking the alkaloid with acetic anhydride (3 ml) and anhydrous sodium acetate (0·1 g) for 1 h before treatment with an excess of aqueous ammonia and extraction with ethyl acetate. After the product had been purified by p.l.c. on silica (in ether; R_F 0·8) it was crystallised from chloroform–methanol; m.p. 191° (much higher than the reported[17] m.p., 169—170°). Protopine was crystallised as above and allocryptopine from methanol; m.p. 157—159° (lit.,[16] 160°).

(*RS*)-Stylopine (50 mg) and (*RS*)-canadine (50 mg) were added to a solution of extract C in methanol and the coptisine and berberine present in it were reduced with borohydride. After partition between ethyl acetate and water, the extracted alkaloids were separated by p.l.c. as above.

Isolation and Degradation of Berberine (3).—The shoots of *Berberis japonica* were macerated with water in a Waring blender and the mixture (1 l) was boiled for 0·5 h. After decanting the extract, the residue was extracted in the same way four times more. The combined extracts were concentrated to 400 ml, diluted with ethanol (400 ml) and, after 0·5 h at 0 °C, the solution was filtered and evaporated. The residue in hot water (60 ml) was mixed with ethanol (500 ml) and a further heavy precipitate was filtered off and the filtrate was evaporated. The residue was fractionated on alumina (neutral, grade I) in chloroform and then an increasing proportion of methanol in chloroform (up to 1 : 4 methanol–chloroform). Evaporation of the yellow fractions gave crude berberine, which was dissolved in water (5 ml) and treated with ammonium chloride to yield berberine chloride (100 mg) (Found: C, 58·5; H, 5·3. Calc for $C_{20}H_{18}ClNO_4$-$2H_2O$: C, 58·9; H, 5·4%). The incorporation of activity from (*RS*)-[*N*-*methyl*-^{14}C, 3-^{14}C]laudanosoline carrying 32% of the activity at the *N*-methyl group was 0·07%. The active alkaloid was further diluted to give material of activity 1·71 × 10^5 disint. per 100 s per mmol.

Phenylmagnesium bromide [from bromobenzene (0·8 g)] in ether (10 ml) was added to a stirred suspension of berberine chloride (200 mg) in dry ether (20 ml) and the mixture was heated under reflux for 4 h. 2N-Hydrochloric acid was then added and the precipitate was collected, washed with water and ether, and fractionated on alumina (20 g; neutral, activity I) in chloroform to give phenyldihydroberberine (110 mg), m.p. 194—196° (from chloroform–methanol) (lit.,[18] 195°).

A solution of this product (95 mg) in 1 : 1 water–acetic acid (10 ml) was treated dropwise at 55—60 °C with potassium permanganate (1·5 g) in water (60 ml). After 5 h, the permanganate and manganese dioxide were destroyed with sodium disulphite and the solution was extracted continuously with ether for 15 h. The extracted material was chromatographed on silica (3 g) in 1 : 9 ether–light petroleum

[15] R. H. F. Manske and W. R. Ashford, in ' The Alkaloids,' ed. R. H. F. Manske, Academic Press, New York, 1954, vol. IV, p. 78.
[16] R. H. F. Manske in ref. 15, p. 147.
[17] J. Gadamer, H. Dieterle, A. Stichel, M. Theissen, and K. Winterfeld, *Arch. Pharm.*, 1924, **262**, 249.
[18] M. Freund and H. Beck, *Ber.*, 1904, **37**, 4673.

(b.p. 60—80°) and the isolated benzoic acid (15 mg) was sublimed at 105° and 760 mmHg to give pure material, m.p. 121—122° (Found: C, 68·6; H, 5·0. Calc. for $C_7H_6O_2$: C, 68·8; H, 5·0%), specific activity 5·8 × 10^4 disint. per 100 s per mmol, corresponding to 34% of the activity in berberine (expected value 32%).

Degradation of Multiply Labelled Reticuline.—The route has been described.[9] Radioactive reticuline (as for experiment 3, Table 2) was diluted to 450 mg. This yielded *trans*-laudanosine methine hydrochloride (320 mg), m.p. 220—221° (lit.,[9] 220°). Oxidation led to veratric acid (151 mg), m.p. 181—182°, and the amino acid which was further degraded to 6-vinylveratric acid (133 mg), m.p. 182—184° (lit.,[9] 183—184°) and trimethylamine picrate (25 mg), m.p. 217—218°. Part of the 6-vinylveratric acid was reduced to 6-ethylveratric acid, m.p. 140—141°. Radioassays are given in Table 1.

Degradation of Labelled Stylopine (Hofmann Route).—(*RS*)-Stylopine (0·1 g) from experiment 1, Table 2, of activity 7·50 × 10^4 disint. per 100 s per mmol was heated under reflux for 2 h with methyl iodide (4·3 ml) and methanol (1·3 ml). The mixture was evaporated and the residue was heated for 3 h with aqueous 20% potassium hydroxide (5 ml) under reflux. Extraction with ether afforded *stylopine methine* (16), m.p. 136—137° (from ethanol); yield 44 mg; activity 7·49 × 10^4 disint. per 100 s per mmol (Found: C, 71·2; H, 5·6; N, 4·2. $C_{20}H_{19}NO_4$ requires C, 71·2; H, 5·7; N, 4·2%); τ 2·76 (1H, q, *J* 11 and 17 Hz, RCH=C), 3·0 and 3·6 (2H each, s and m, aryl H), 4·05 (4H, s, OCH_2O), 4·52 and 4·81 (each 1H, dd, *J* 1·5 and 11 Hz, C=CH_2), 5·6—7·4 (5H, complex), and 7·80 (3H, s, NMe).

The foregoing methine (9·1 mg) in t-butyl alcohol (1 ml), freshly distilled from sodium, and water (1 ml) was stirred at 18° while osmium tetraoxide solution (0·01 ml; 40 mg ml^{-1}) in t-butyl alcohol was added, followed by sodium periodate (27 mg). After 5 h, the mixture was poured into saturated aqueous arsenous oxide (5 ml), and this mixture was extracted with ether (3 × 5 ml). The aqueous layer was adjusted with potassium carbonate to pH 8·5 and was mixed with dimedone (32 mg) before being adjusted to pH 6. After 16 h, the formaldehyde derivative (8·6 mg) was collected and filtered in chloroform through alumina (2 g). The product from the eluate, m.p. 191—192° (from ethanol) (5·3 mg), was identified by comparison with authentic material; activity 3·77 × 10^4 disint per 100 s per mmol (50% of original). A second run using 25·2 mg of methine gave the final derivative (15·7 mg) of activity 4·06 × 10^4 disint. per 100 s per mmol (54% of original).

A solution of stylopine methine (100 mg) in methanol (25 ml) was shaken with hydrogen and platinum oxide (20 mg) at 18° and 760 mmHg for 3 h (uptake complete). The residue from evaporation of the filtered solution crystallised from ethanol to give *stylopine dihydromethine* (17), m.p. 132—133° (44 mg) (Found: C, 70·4; H, 6·1. $C_{20}H_{21}NO_4$ requires C, 70·8; H, 6·2%).

The foregoing dihydromethine (19·7 mg) was treated as above with osmium tetraoxide and periodate. Radioactive formaldehyde dimedone derivative (5·865 mg) was added in the work-up before the adjustment to pH 6. The pure derivative isolated (6 mg) had activity 298 disint. per 100 s per mg and that added had activity 453 disint. per 100 s per mg, corresponding to formation of 0·18 mol. equiv. of formaldehyde.

Degradation of Labelled Stylopine (Grignard Route).—(a) *Phenyl series.* A solution of stylopine (140 mg) and sodium acetate (750 mg) in acetic acid (7·5 ml) and ethanol (50 ml) was heated on a steam-bath and treated in portions with ethanolic iodine until an excess was present. Coptisine iodide (168 mg) crystallised from the cooled solution; it was identified by conversion into the chloride [with Amberlite IRA-400 resin (Cl^-)] and comparison with natural coptisine chloride.

To phenylmagnesium bromide [from bromobenzene (1·6 ml)] in ether (40 ml) was added coptisine iodide (168 mg), and the mixture was kept at 45 °C for 4 h and then treated with water and 2N-hydrochloric acid. Part (30 mg) of the precipitate (154 mg) was dissolved in ethanol–acetic acid and the solution was basified with ammonia to give *phenyldihydrocoptisine* (20), m.p. 195—197° (Found: C, 75·3; H, 4·7. $C_{25}H_{19}NO_4$ requires C, 75·5; H, 4·8%). This product (16 mg) was dissolved in methanol (25 ml) and reduced with borohydride. The colourless solution was evaporated and the residue from chloroform–ethanol gave *phenyltetrahydrocoptisine* (21), m.p. 237—239° (14 mg) (Found: C, 75·2; H, 5·2. $C_{25}H_{21}NO_4$ requires C, 75·2; H, 5·3%).

Oxidation of phenyldihydrocoptisine (124 mg) with permanganate essentially as above for phenyldihydroberberine gave benzoic acid (8·1 mg).

(b) *Benzyl series.* Coptisine iodide (246 mg) was added to a solution of benzylmagnesium bromide [from benzyl chloride (1·16 ml) and magnesium (270 mg)] in ether (30 ml) and the mixture was heated at 45 °C for 5 h. 6N-Hydrochloric acid was then added and the aqueous phase was basified with ammonia to give *benzyldihydrocoptisine* (22) (204 mg), m.p. 165—166° (from acetone–methanol) (Found: C, 76·0; H, 5·1; N, 3·3. $C_{26}H_{21}NO_4$ requires C, 75·9; H, 5·1; N, 3·4%). Reduction of this product (200 mg) with borohydride essentially as in (a) gave *benzyltetrahydrocoptisine* (23) (190 mg), m.p. 164—165° (from chloroform–methanol) (Found: C, 75·3; H, 5·6; N, 3·4. $C_{26}H_{23}NO_4$ requires C, 75·5; H, 5·6; N, 3·4%).

Benzyltetrahydrocoptisine (180 mg) and methyl iodide (2·5 ml) in a sealed tube were heated in boiling acetone for 4 days. The residue from evaporation was heated under reflux for 2·5 h with methanolic potassium hydroxide (360 mg in 2·75 ml) before water (25 ml) was added. Extraction with ether gave a gum which was treated in methanol with dry hydrogen chloride to give the *methine hydrochloride monohydrate* (80 mg), m.p. 173—175° (from methanol) (Found: C, 67·3; H, 5·7; N, 3·1. $C_{27}H_{26}ClNO_4,H_2O$ requires C, 67·3; H, 5·85; N, 2·9%).

The base (58 mg), recovered as usual from the foregoing hydrochloride, was heated at 100 °C for 3 h in acetic acid (1·17 ml) with sodium dichromate (46 mg in 0·58 ml of 1 : 1 water–acetic acid). Water (1 ml) was then added, the mixture was adjusted with sodium carbonate to pH 7·5, and extraction with ether (stage A) gave a residue which was chromatographed on alumina (addition in 1 : 1 chloroform–benzene; elution with benzene). The product from yellow fractions crystallised from methanol to give 3,4-*methylenedioxy*-2-*styrylbenzaldehyde* (26), m.p. 95—96° (Found: C, 76·0; H, 4·8. $C_{16}H_{12}O_3$ requires C, 76·2; H, 4·8%), $\nu_{max.}$ 1676, 1612, 1580, and 1455 cm^{-1}.

The aqueous phase from stage A was basified with sodium hydroxide, and extraction with ether gave hydrastinine (12 mg), m.p. 110—112° (from ethyl acetate), identified by comparison with authentic material prepared from hydrastine.

Site of ^3H *Label in Stylopine from Experiment* 1, *Table* 2.—

Oxidation of the stylopine (20 mg) with iodine as above gave coptisine iodide (20 mg), which was converted into the chloride. Recrystallisation from aqueous methanol gave pure chloride, and this (9·1 mg) was reduced in 1 : 10 water–methanol (11 ml) with borohydride to give (*RS*)-stylopine (identified by i.r.), which was recrystallised twice from benzene–methanol (yield 5·4 mg).

(+)-(S) *and* (−)-(R)-[N-methyl-^{3}H, 3-^{14}C]*Reticulines*.—[^{3}H]Methyl iodide (29 mg; 25 mCi) was transferred by a vacuum line into a tube containing 7-benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxyisoquinoline [19] (383 mg) in ethyl acetate (10 ml). The tube was sealed and kept for 1 week at 20°, and then the contents were evaporated. Methyl iodide (2 ml) and ethyl acetate (10 ml) were added to the residue and, after 2 days, the crystals (405 mg) were collected, suspended in propan-2-ol (12 ml) and treated with borohydride (50 mg). After 3 days, the basic product was worked up as usual to give a gum (315 mg), which afforded the crystalline picrate from which the pure (*RS*)-[N-*methyl*-^{3}H]-*OO*-dibenzylreticuline (101 mg; 6·22 mCi) was recovered by percolation in chloroform over alumina.

This (24·6 mg; 1·51 mCi) was mixed with (*RS*)-[3-^{14}C]-*OO*-dibenzylreticuline [20] (76·5 mg; 0·145 mCi) and resolved [8] to give the (+)-(*S*)-base (40·5 mg), m.p. 91—92°, $[\alpha]_D$ +43·4° (*c* 1·0 in $CHCl_3$). The (−)-(*R*)-base (32·7 mg) had m.p. 91—93°, $[\alpha]_D$ −42·6° (*c* 1·0 in $CHCl_3$). *O*-Debenzylation of both bases was carried out with hot hydrochloric acid as previously.[8]

Rearrangement of N-*Oxides*.[20]—Part (*ca.* 1 mg) of the foregoing (*RS*)-[N-*methyl*-^{3}H, 3-^{14}C]reticuline was methylated with an excess of diazomethane and the product was diluted with radioinactive (*RS*)-laudanosine (total product 83 mg). This was mixed with [N-*methyl*-^{14}C]laudanosine [4] (130 mg) to give a ^{3}H : ^{14}C ratio of 1·83 : 1 (calculated w.r.t. *N*-methyl group only). The base and 30% hydrogen peroxide (0·2 ml) in acetone (5 ml) were heated at 60—70 °C for 30 min, the peroxide was then decomposed on platinum wire over 2 h at 60 °C, and the solution was evaporated. The residue in water (20 ml) was added to a solution of iron(III) nitrate nonahydrate (1·2 g) and oxalic acid (0·5 g) in water (10 ml) and the mixture was heated in a nitrogen stream at 130 °C for 2 h. The gas stream was passed through a solution of dimedone (0·2 g) in water (60 ml) and the formaldehyde dimedone derivative which separated during 1 day was collected and recrystallised from aqueous methanol; yield 7·7 mg; m.p. 193—194°; ^{3}H : ^{14}C ratio 1·83 : 1.

3-*Benzyloxy*-4-*methoxy*-NN-*dimethylphenethylamine*.— 3-Benzyloxy-4-methoxyphenethylamine [21] (4·36 g), formalin (3·8 ml), and formic acid (0·92 ml) were heated at 85—90 °C for 3 h while the pH was maintained at 7·2 by addition of formic acid in portions. After addition of water (50 ml), the solution was worked up for base as usual to give a gum (2·15 g) which slowly crystallised; m.p. 54—55°. The *picrate* (from ethanol) had m.p. 148—150° (Found: C, 55·9; H, 5·0; N, 10·8. $C_{24}H_{26}N_4O_9$ requires C, 56·0; H, 5·1; N, 10·9%).

6-*Benzyloxy*-1,2,3,4-*tetrahydro*-7-*methoxy*-2-*methylisoquinoline*.—3-Benzyloxy-4-methoxyphenethylamine (1·13 g), formic acid (1·18 ml), and formalin (1·25 ml) were heated for 6 h at 100 °C and the basic product was isolated as above; m.p. 83—84°. Its *picrate* (905 mg) (from ethanol) had m.p. 164—166° (Found: C, 56·4; H, 4·7; N, 10·9. $C_{24}H_{24}N_4O_9$ requires C, 56·2; H, 4·7; N, 10·9%).

3-*Hydroxy*-4-*methoxy*-NN-*dimethylphenethylamine*.— The corresponding *O*-benzyl ether (675 mg) was heated with 6N-hydrochloric acid (30 ml) at 130 °C under nitrogen for 1 h and the solution was evaporated. Crystallisation of the residue from propan-2-ol gave the *base hydrochloride* (404 mg), m.p. 155—156° (Found: C, 56·9; H, 7·9; N, 5·8. $C_{12}H_{18}NO_2$,HCl requires C, 56·6; H, 7·8; N, 6·0%).

N-*Oxide Rearrangement Catalysed by Chromate*.—The *N*-oxide of 3,4-dimethoxy-*NN*-dimethylphenethylamine (236 mg) was prepared by standard methods and characterised as the picrate, m.p. 153—156° (lit.,[20] 156—158°) (Found: C, 47·7; H, 4·8; N, 12·2. Calc for $C_{18}H_{22}N_4O_{11}$: C, 47·6; H, 4·9; N, 12·3%). A solution of the *N*-oxide in water (5 ml) was added to potassium chromate (0·25 g) in water (4 ml) and the mixture was adjusted to pH 5 with acetic acid. After 3 days at 20 °C, the basic products (106 mg) were extracted and treated in 2·5N-sodium hydroxide (3 ml) with benzenesulphonyl chloride (0·5 ml) to yield N-(3,4-*dimethoxyphenethyl*)-N-*methylbenzenesulphonamide* (147 mg), m.p. 68—69° (from aqueous ethanol) (Found: C, 60·8; H, 6·4; S, 9·5. $C_{17}H_{21}NO_4S$ requires C, 60·9; H, 6·3; S, 9·4%). Rearrangements catalysed by Fe^{3+}–oxalic acid or Fe^{3+}–tartaric acid gave similar results.

An authentic sample of the product was prepared as follows. Furfuraldehyde (0·6 g) and 3,4-dimethoxyphenethylamine (0·5 g) were mixed, and after 2 h the generated water was removed by codistillation with benzene (50 ml). Methyl iodide (5 ml) was added to the residue and after 20 h at 20 °C N-*furfurylidene*-3,4-*dimethoxyphenethylamine methiodide* was collected (1·07 g); m.p. 170—171° (Found: C, 47·7; H, 5·0; N, 3·7. $C_{16}H_{20}INO_3$ requires C, 47·9; H, 5·0; N, 3·5%). This (199 mg) was heated at 80 °C for 30 min with 2N-sulphuric acid (10 ml) and the basic product was converted as above into its benzenesulphonyl derivative, m.p. 90—91° (from aqueous ethanol). A solution of the product, m.p. 68—69°, from the *N*-oxide rearrangement was dissolved in ethanol and seeded with a trace of the material m.p. 90—91°. The crystals formed had m.p. and mixed m.p. 90—91° thus demonstrating dimorphism.

(−)-(S)-[1,12-^{3}H]*Scoulerine*.—A solution of (−)-(*S*)-scoulerine (10 mg) in methanol was titrated to pH 8·5 with methanolic 5% tetramethylammonium hydroxide and was then evaporated to dryness in a Carius tube. Tritiated water (0·02 ml; 5 Ci ml^{-1}) and methanol (0·03 ml) were added, and the tube was sealed and heated at 100 °C for 36 h. The residue from evaporation was purified by repeated (4 times) dissolution in methanol and evaporation, and was then dissolved in a slight excess of hydrochloric acid. Radioinactive (−)-scoulerine (30 mg) was added and the residue from evaporation was crystallised from methanol–ether to yield the labelled scoulerine hydrochloride (36 mg), m.p. 196—200° (decomp.), total activity 1·05 mCi.

Dilution Analysis for Scoulerine in C. majus *Plants*. The aqueous solution containing the total alkaloids from plants from experiment 4, Table 2, was adjusted to pH 8·4 and extracted five times with chloroform. In a solution of the extracted material (20 mg) in acetone was dissolved radioinactive (*RS*)-scoulerine picrate (50 mg); alumina (2 g) was added and the solvent was evaporated off. The solid was

[19] A. R. Battersby, R. Binks, R. J. Francis, D. J. McCaldin, and H. Ramuz, *J. Chem. Soc.*, 1964, 3600.

[20] For related work see P. A. Bather, J. R. Lindsay Smith, and R. O. C. Norman, *J. Chem. Soc.* (*C*), 1971, 3060; J. R. Lindsay Smith, R. O. C. Norman, and A. G. Rowley, *J.C.S. Perkin I*, 1972, 228.

[21] R. D. Haworth, J. B. Koepfli, and W. H. Perkin, *J. Chem. Soc.*, 1927, 548.

packed on the top of a column of alumina (12 g), and chloroform eluted the scoulerine (39 mg). It was purified by recrystallisation of the picrate (36 mg) from methanol and then the base (23 mg) was recovered by the alumina–chloroform method. Repetition of picrate formation, recrystallisation, and recovery of the base gave (*RS*)-scoulerine of constant activity (16 mg), m.p. 192—196°.

This base (13·5 mg) in methanol (2 ml) and ether (20 ml) was treated for 24 h with an excess of diazomethane and the product was chromatographed in benzene on alumina (2 g). The homogeneous main product gave (in ether with dry HCl) (*RS*)-tetrahydropalmatine hydrochloride, m.p. 217—218° (lit.[22] 215°), from which the base was isolated as usual; m.p. 150—151° (lit.,[22] 147°).

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[22] R. Robinson and S. Sugasawa, *J. Chem. Soc.*, 1931, 3163.