PERKIN 1

Biosynthesis. Part 30.[1] Colchicine: studies on the ring expansion step focusing on the fate of the hydrogens at C-4 of autumnaline

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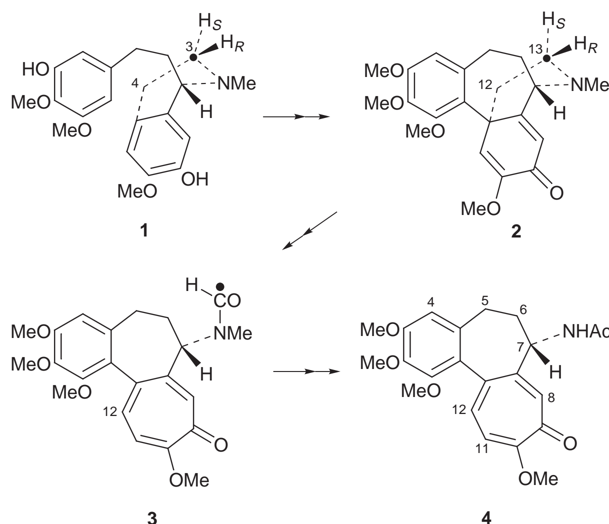
Autumnaline 1 is a key biosynthetic precursor of colchicine 4. Syntheses are described of autumnalines that are labelled stereospecifically with tritium at C-4 of their isoquinoline ring. This corresponds to C-12 of the later intermediate dienone 2. Incorporation experiments with the labelled autumnalines using *Colchicum autumnale* plants show that (a) both H_R and H_S at C-4 of 1 are fully retained as the dienone is generated, (b) there is stereospecific loss of H_S from C-12 of the dienone during the ring expansion process by which the tropolone ring of colchicine is formed and (c) also, there is partial loss (*ca.* 20%) of the ^{3}H label from the H_R position at C-12. These results are rationalised by a proposal for the biosynthesis of colchicine that involves a cyclopropane intermediate in the ring expansion step plausibly formed by a radical process.

Introduction

The Introduction to the preceding paper[1] described with references how colchicine **4** is biosynthesised from the isoquinoline (1*S*)-autumnaline **1**, Scheme 1, which undergoes phenolic oxidative coupling and *O*-methylation to form the dienone *O*-methylandrocymbine **2**. Then, by an intriguing process, the dienone ring is expanded to generate the tropolone system of the *Colchicum* alkaloids with C-12 of **2** becoming C-12 of *N*-formyldemecolcine **3**. Further steps modify the groups attached to the nitrogen of **3** finally to generate colchicine **4**. Scheme 1 also shows the outcome of experiments reported in the preceding paper[1] which demonstrated that the ring expansion process involves the stereospecific removal of H_S from C-13 of the dienone **2** whereas H_R at C-13 of **2** is retained in the formyl group of *N*-formyldemecolcine **3**. The present paper decribes our complementary studies on C-4 of autumnaline **1** corresponding to C-12 of the dienone **2**.

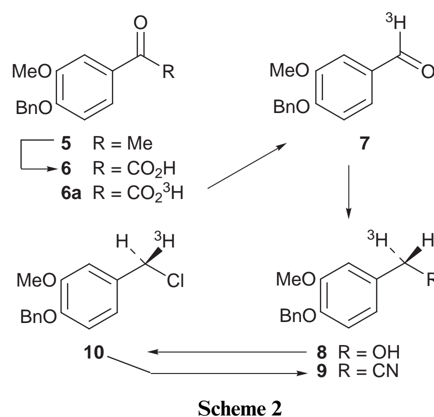


Scheme 1

Results and discussion

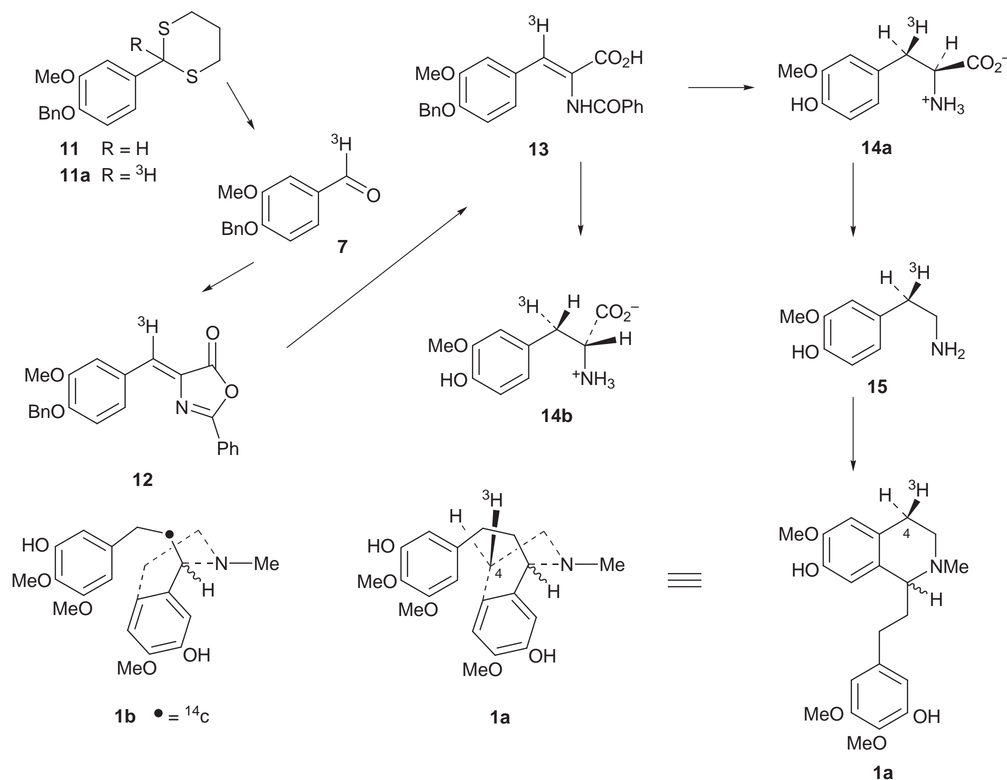
Syntheses of autumnaline 1 labelled stereospecifically with ^{3}H at C-4: exploratory studies

The first plan involved generation of the required centre, made chiral by isotopic substitution at C-4 of autumnaline **1**, by reducing the [*formyl*-^{3}H]aldehyde **7** with liver alcohol dehydrogenase[cf. 1] to yield the aryl alcohol **8** (Scheme 2). The labelled aldehyde was prepared by permanganate oxidation[2] of the known ketone[3] **5** to afford the glyoxylic acid **6** which by exchange with tritiated water gave the [^{3}H]acid **6a**. This was decarboxylated by heating with benzoic anhydride in pyridine[4] and the resultant [*formyl*-^{3}H]aldehyde† **7** was enzymically reduced to the (*S*)-[α-^{3}H$_1$]alcohol **8**. Since the conversion of 1-phenylethanol into the corresponding chloride using phosphorous oxychloride and pyridine occurs with a high degree of inversion,[6] this method was applied to **8** aiming to form mainly the chloride **10**. The configurational purity of this product was to have been subsequently checked but, as will be seen, this became unnecessary. Displacement of chloride from **10** by



Scheme 2

† A better method for the preparation of this aldehyde is given later in this paper. Furthermore, an even simpler approach to [*formyl*-^{3}H]aldehydes was subsequently used for several other syntheses (see *e.g.* ref. 5).



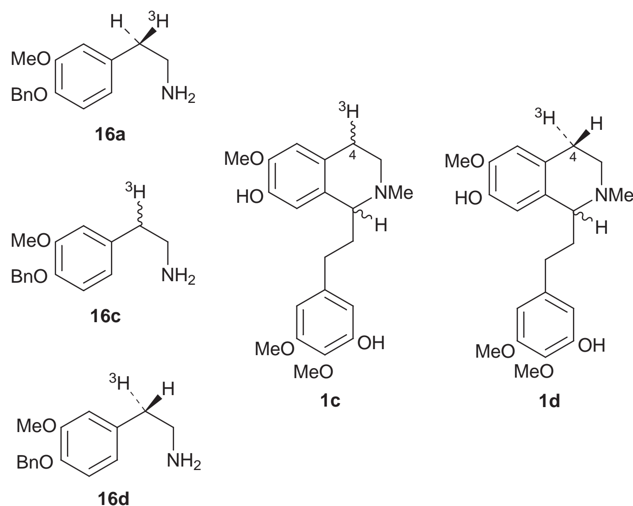
Scheme 3

cyanide ion was carried out under conditions to favour S_N2 over S_N1 reactions using benzyltrimethylammonium cyanide which is soluble in acetone. However, the nitrile **9** so formed had lost over 90% of the tritium present in the chloride **10**. Clearly, the hydrogens of the methylene group in the nitrile **9** had undergone base catalysed exchange with the medium during its preparation; cyanide ion or trimethylamine from decomposition of the quaternary cyanide are possible bases involved. Loss of tritium also signalled loss of stereochemical integrity so this approach was unsuitable, but it taught us valuable lessons.

The second approach started by quenching the anion from the dithiane **11** with tritiated water to afford the [^{3}H]dithiane **11a**, Scheme 3. Cleavage of the dithiane gave the [*formyl*-^{3}H]aldehyde **7** which was converted into the oxazolone **12**; such oxazolones have been proved[8,9] to have the illustrated (*Z*)-configuration. Base catalysed ring-opening of **12** afforded the (*Z*)-2-(benzoylamino)propenoic acid **13** which was hydrogenated over palladium to saturate the double bond and cleave the *O*-benzyl group. Hydrolysis of the *N*-benzoyl group then afforded a racemic mixture of (2*R*,3*S*)-3-methoxy[β-^{3}H$_1$]-tyrosine **14b** and (2*S*,3*R*)-3-methoxy[β-^{3}H$_1$]tyrosine **14a**. These configurations arise because the reduction of the double bond is *syn*-stereospecific.[10]

Incubation of the racemate with tyrosine decarboxylase as in the preceeding paper[1] brought about decarboxylation of **14a** to yield the amine **15** leaving the readily separable **14b** unchanged. Finally, the amine **15** was converted into (1*RS*,4*R*)-[4-^{3}H$_1$]autumnaline **1a** by the previously developed route.[1,6] (1*RS*)-[9-^{14}C]Autumnaline[7] **1b** was added to the foregoing ^{3}H-labelled material to act as internal standard and the doubly labelled material was injected into the seed capsules of *Colchicum autumnale* plants. These were allowed to grow for *ca.* 2 weeks and the colchicine then isolated from them was labelled at a level corresponding to 2.5% incorporation of the precursor. The ^{3}H : ^{14}C ratio showed 75% retention of tritium.

This was a puzzling result. The work described in the preceeding paper[1] showed that the enzymic resolution gave material of high configurational purity and there was no reason to suppose that the outcome would be any different for the present experiments. Nevertheless, to have a result only from the (4*R*)-system **1a** was insufficient because it could be interpreted in several ways: (a) the configurational purity at C-4 of **1a** may only be 75% (*R*) : 25% (*S*) or (b) the configurational purity is high but there is non-stereospecific removal of hydrogen from C-12 of the dienone **2** corresponding to C-4 of autmnaline **1**; in that case there would be *ca.* 80% retention of tritium because of the kinetic isotope effect or (c) other as yet unknown events are occurring. It was clear that *both* the (4*R*)-autumnaline **1a** and the (4*S*)-isomer **1d** were needed, and that the amine precursors **16a** and **16d** had to be prepared by a



method which would allow their configurational purity to be determined rigorously and independently.

Synthesis of (1*RS*,4*R*)-[4-^{3}H$_1$]autumnaline 1a and (1*RS*,4*S*)-[4-^{3}H$_1$]autumnaline 1d and their incorporation into colchicine by *C. autumnale* plants

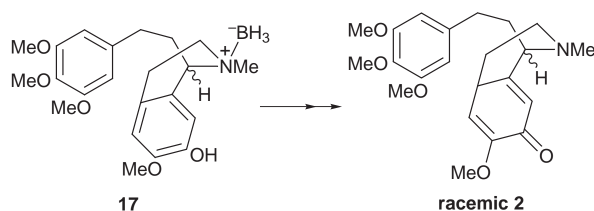
In fact, syntheses matching the above requirements were becoming available from research in Cambridge[5] aimed at

Table 1 Incorporation of labelled autumnalines into *Colchicum* plants

	$^3H:^{14}C$ Ratios and [^3H]-retentions (%)		
	(4*R*)-[4-$^3H_1$,9-^{14}C] Autumnaline **1a + 1b**	(4*RS*)-[4-$^3H_1$,9-^{14}C] Autumnaline **1c + 1b**	(4*S*)-[4-$^3H_1$,9-^{14}C] Autumnaline **1d + 1b**
O,*O*-Dibenzylautumnaline	10.6	10.4	10.0
Autumnaline	10.6	10.4	10.0
O-Methylandrocymbine **2**	10.2 (96)	10.3 (99)	9.9 (99)
Incorporation	0.32%	0.56%	0.24%
Colchicine **4**	8.3 (79)	4.1 (39)	0.02 (0.2)
Incorporation	34%	3.5%	6.4%

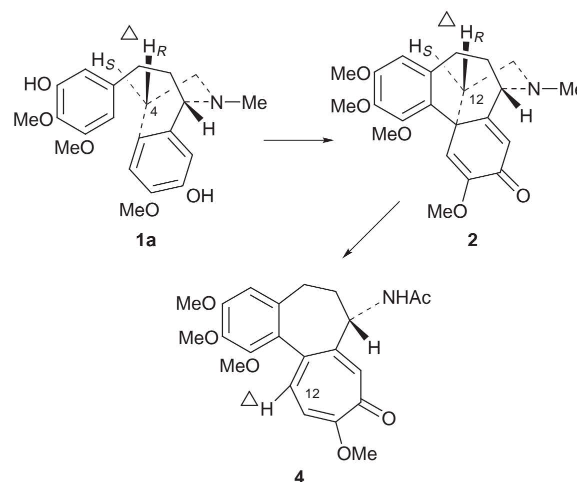
determination of the stereospecificity of dopamine β-hydroxylase. Not only were both the (2*R*)-amine **16a** and the (2*S*)-amine **16d** shown from work in the analogous ^2H-series to be enantiomerically pure but the results obtained using dopamine β-hydroxylase[5] fully confirmed these findings. Accordingly, the (2*R*)-amine **16a** was converted as earlier[6] into (1*RS*,4*R*)-[4-$^3H_1$]autumnaline **1a** and the (2*S*)-amine **16d** similarly afforded (1*RS*,4*S*)-[4-$^3H_1$]autumnaline **1d**. The racemic [^3H]amine **16c** was also available from earlier work[1] and this was used for the synthesis of (1*RS*,4*RS*)-[4-$^3H_1$]autumnaline **1c**. Each of the three [^3H]-labelled autumnalines, in their *O*,*O*-dibenzyl protected form, were mixed with an appropriate quantity of similarly protected (1*RS*)-[9-^{14}C]autumnaline **1b** to give a suitable $^3H:^{14}C$ ratio. The three doubly labelled samples were recrystallised to constant $^3H:^{14}C$ ratio and specific activity and were then debenzylated by hydrogenolysis. The three samples of autumnaline hydrochloride so produced were similarly purified and the $^3H:^{14}C$ ratios remained unchanged demonstrating their chemical and radiochemical purity.

These three autumnalines, the (4*R*)-material **1a + 1b**, the (4*RS*)-sample **1c + 1b** and the (4*S*)-base **1d + 1b**, were separately injected into the seed capsules of *Colchicum autumnale* plants at *ca.* 1 mg for each capsule and the plants were allowed to develop for a further 2 weeks. They were then worked up for colchicine **4** and *O*-methylandrocymbine **3**. The latter is present in the plants only in minute amounts (*ca.* 40 µg in a full sized plant including the corm) so synthetic racemic *O*-methylandrocymbine (as **2**), see below, was added during the extraction procedure to act as carrier material. The various samples of the two alkaloids were rigorously purified to constant specific activity and $^3H:^{14}C$ ratio. The results are collected in Table 1 but before discussing them, the preparation of synthetic racemic *O*-methylandrocymbine (as **2**) should be outlined. This was done by the method of Schwartz *et al.*[11] involving oxidative coupling of the aryl rings of the isoquinoline borane complex **17**, Scheme 4. The isoquinoline itself (as **17** lacking borane) was synthesised essentially by the method of Brossi *et al.*[12]



Scheme 4

The results in Table 1 show that, as we expected, both hydrogens at C-4 of autumnaline **1** are unaffected as it is converted by the plants into *O*-methylandrocymbine **2**. Furthermore, they demonstrate that as the dienone ring of **2** undergoes expansion to the tropolone ring of colchicine **4**, there is a stereospecific total loss of H_S from C-12 of **2** which becomes C-12 of colchicine, Scheme 5.

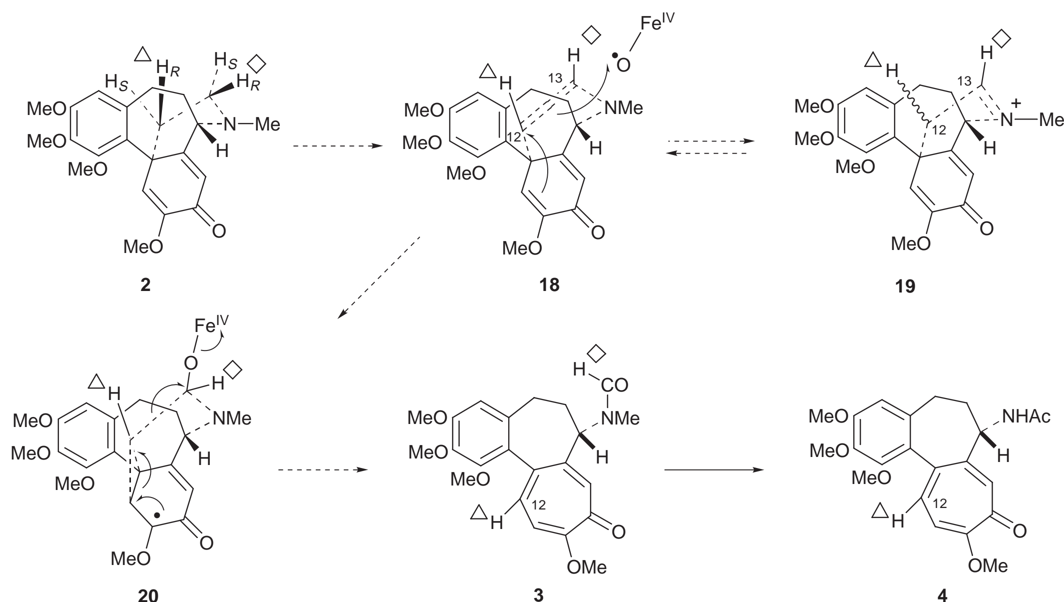


Scheme 5

It is not immediately clear what has affected H_R at C-12 of the dienone **2** to lead to a retention of tritium of 79%, a result that agrees with the first study outlined above where the [^3H]-retention was 75% from a sample of the (4*R*)-precursor **1a** synthesised in a different way. However, retention of most of the tritium at the H_R position of C-12 eliminates an early idea[6] that the ring expansion might be initiated by hydroxylation of this centre. Stereomodels show that the double bond of the dienone can only interact with C-12 in the possible loss of such an activated hydroxy group if the latter occupies the site held initially by H_R. Since such hydroxylations at saturated carbon occur with retention of configuration (see ref. 5 for leading refs.), there would have been complete loss of H_R which is not the case.

One possibility for the loss of *ca.* 20% of the tritium might be by some exchange process from C-12 of colchicine **4** during the isolation steps. Accordingly, the doubly labelled colchicine from the experiment with (4*RS*)-[4-$^3H_1$]autumnaline **1c** having a $^3H:^{14}C$ ratio of 4.1 was reworked through the entire isolation procedure and then the $^3H:^{14}C$ ratio was redetermined; it was unchanged at 4.1. The partial loss of tritium from the H_R position is thus due to some real effect in the biosynthetic transformation of **2** into **4**. This conclusion is supported by the fact that the result from the (4*RS*)-experiment (39% retention) is the mean of the values from the (4*R*)-, and (4*S*)-precursors **1a** and **1d**.

The importance of having this full set of retention values is emphasised by looking back at an early result[13] of 44% retention from the (4*RS*)-precursor **1c** which, whilst close to the foregoing value is also not far away from 50% and so *on its own* could indicate a straightforward stereospecific process at C-12 of the dienone **2**. In that case, the complete loss of H_S from C-12 would be mirrored by complete retention of H_R which is not observed. The conclusion is that after the initial 100% retention of H_R, something further happens to cause the *ca.* 20% loss. In summary, the results from this paper and the preceding one[1] are that there is (a) complete loss of H_S from both C-12 and C-13 of the dienone **2** as it is converted into *N*-formyldemecolcine



Scheme 6

3 *en route* to colchicine **4** and (b) complete retention of H_R originally at C-13 but loss of over 20% of H_R from C-12. The relevant hydrogen atoms are marked in Scheme 6. The following discussion and biosynthetic scheme provides a rationalisation of these findings.

A key feature is the proposal that the ring expansion step involves a cyclopropane shown in Scheme 6 as a transient radical **20**. Closely related cationic chemistry is well known [14,15] in a variety of systems. A plausible earlier intermediate is the enamine **18** which will be in chemical equilibrium with the quaternised imine **19**. This will lead to exchange of hydrogen at C-12 with the medium, neatly explaining loss of part of H_R from C-12 following its initial complete retention; experimental support is available. Thus, the racemic form of the enamine **18** has been synthesised [16] and found to be unstable. Repetition of that synthesis provided a sample of the enamine **18** which we also found to be labile. Nevertheless, it was possible to demonstrate proton exchange at C-12 by ^{1}H NMR spectroscopy. The pair of doublets at δ 5.03 and 5.92 in the spectrum of **18** are assigned to the protons at C-12 and C-13, respectively. When **18** was kept in solution in tetradeuteriomethanol at 4 °C, the signal at δ 5.03 had entirely disappeared after 16 h and that at δ 5.92 had appropriately become a singlet. Mass spectroscopic analysis of the recovered enamine **18** demonstrated the incorporation of a single atom of deuterium.

The two hydrogen atoms that are enzymically removed from the ethanamine bridge of the dienone **2** (H_S at both centres) are *syn*-related and lie well exposed. Dehydrogenases are known [17] that bring about overall *syn*-removal of two hydrogens from adjacent carbons so one such enzyme could be involved here to form the enamine **18** as precursor of the imine **19**. However, there are numerous examples of the enzymic oxidation of amines to imines so the order could be reversed. In that case, a basic centre would be needed on the enzyme, judiciously placed to remove specifically H_S from C-12 of the imine **19** the first time the double bond migrates in the imine to enamine tautomerisation. The exchange reported above could then occur either by the enzyme undergoing some exchange with the medium or by the imine–enamine system having a significant lifetime off the enzyme before being further transformed.

The discussion so far of Scheme 6 is on reasonably firm ground by virtue of experimental support and close analogies. An attractive way to generate the cyclopropane **20** is to involve a P-450 enzyme in a radical process [18] as illustrated in Scheme 6. Models show that the p-orbitals of the two double bonds of **18** taking part in forming the cyclopropane are well positioned for strong interaction. The illustrated radical in **20** is a favourable one and also, being a cyclopropylcarbinyl radical, would rapidly ring open leading directly to *N*-formyldemecolcine **3** (for references to radical processes in biological systems, see ref. 18). Several rather closely related variations on this theme can be envisaged *e.g.* formation by a P-450 enzyme of the epoxide of the enamine **18** which could proceed through similar cationic chemistry to the tropolone **3**. However, the mechanism in Scheme 6 is in our view the most plausible.

Conclusions

The results from this set of four papers and from all the earlier ones from our group (refs. collected in refs. 1 and 5) delineate in almost complete detail the biosynthetic pathway by which colchicine **4** and its relatives are built in *Colchicum* plants. Prior to 1966,[19] colchicine had been a mystery apparently unrelated to other natural products. The route that is followed was totally unexpected but the outcome was that colchicine fell perfectly into place and the picture has been substantially filled out since that time. These successes show the power of the multiple labelling approach used here in combination with stereospecific labelling. Essentially everything that can be done in this way has been completed and a few remaining mechanistic problems demand research on the plant enzymes. For example, is a P-450 enzyme needed for the ring expansion step as proposed in Scheme 6? Knowledge of the pathway to colchicine **4** provided by our work should help the enzymologists in that the precursor and product, and likely intermediates, are known for the enzymic steps of interest.

Experimental

General

General directions are given in Part 26 [20] except that an improved procedure for isolation and purification of the alkaloids is described later in this paper.

2-Oxo-2-(4-benzyloxy-3-methoxyphenyl)ethanoic acid 6

1-(4-Benzyloxy-3-methoxyphenyl)ethan-1-one **5** (3 g) was suspended in water (300 cm^3) containing sodium hydroxide (30 cm^3, 1 mol dm^{-3}) and stirred at 70 °C while potassium permanganate (10 g) in water (500 cm^3) was added dropwise during 2.5 h. After further heating for 1.5 h, the cooled mixture was filtered and the filtrate was acidified with hydrochloric acid, then extracted with dichloromethane (5 × 200 cm^3). The

extracted material (2.8 g) was crystallised from dichloromethane–hexane to afford the α-keto acid **6** (1.79 g, 57%) as needles (Found: C, 67,4; H, 5.0. $C_{16}H_{14}O_5$ requires C, 67.1; H, 4.9%); ν_{max}/cm^{-1} 1675, 1710 ($COCO_2H$).

4-Benzyloxy-3-methoxy[*formyl*-^{3}H]benzaldehyde 7

A solution of the foregoing keto acid **6** (286 mg) in dry dioxane (3.5 cm^3) containing tritiated water (10 mg; 50 mCi) was kept in a sealed tube at room temperature for 2 days. The solvent and tritiated water were removed and collected by freeze sublimation and more of **6** (286 mg) was dissolved in the distillate, then treated and recovered in the same way.

Benzoic anhydride (482 mg), dry benzene (3.8 cm^3) and dry pyridine (1.7 cm^3) were added to the first batch of tritiated **6a** and heated at reflux under nitrogen for 13 h. The solution was evaporated and the residue was chromatographed on alumina first with benzene–light petroleum (1 : 1) followed by benzene which eluted **7** (141 mg). Crystallisation from diisopropyl ether gave pure **7** (111 mg; 1.26×10^{-2} mCi mg^{-1}) shown to be identical to authentic unlabelled material.

The decarboxylation was repeated on the second batch of tritiated **6a** to give more of [*formyl*-^{3}H]-**7** (81 mg; 1.24×10^{-2} mCi mg^{-1}). The material from the two mother liquors was combined and recrystallised to provide another crop of product (26 mg).

(*S*)-4-Benzyloxy-3-methoxy[α-$^3H_1$]benzyl alcohol 8

Liver alcohol dehydrogenase (0.5 cm^3; water suspension containing 5 mg), nicotinamide adenine dinucleotide (30 mg) and ethanol were mixed in phosphate buffer (300 cm^3; 10 mmol dm^{-3}; pH 7.4). The foregoing **7** (54 mg; 1.26×10^{-2} mCi mg^{-1}) in dioxane (4.4 cm^3) was then added and the resultant milky solution was stirred at room temperature for 5 h. The then clear solution was extracted with diethyl ether (4 × 100 cm^3) to yield a gum (55 mg). Purification by PLC using 1 : 19 ethanol–benzene and crystallisation of the product from dichloromethane–light petroleum gave the [α-$^3H_1$]benzyl alcohol **8** (38 mg; 1.28×10^{-2} mCi mg^{-1}) identical to authentic unlabelled material.

4-Benzyloxy-3-methoxy[α-$^3H_1$]benzyl chloride

The foregoing alcohol (220 mg; 9.2×10^6 dis 100 sec^{-1} mmol^{-1}) was dissolved in dry chloroform (1.08 cm^3) and dry pyridine (0.11 cm^3) and redistilled phosphorus oxychloride (0.08 cm^3) were added. After the mixture had been kept at −10 °C for 5 h, it was poured onto ice–water (25 cm^3) and extracted with diethyl ether (3 × 25 cm^3). Chromatography of the extracted material on alumina (10 g) using diethyl ether–light petroleum (1 : 9) gave the chloride which crystallised from light petroleum (162 mg; 9.1×10^6 disintegrations per 100 s per mmol) shown to be identical to authentic unlabelled material. This product has mainly the (*R*)-configuration **10**.

4-Benzyloxy-3-methoxy[α-$^3H_1$]benzyl cyanide (as 9)

Trimethylbenzylammonium hydroxide (15 cm^3; 40% w/w solution in water) in water (50 cm^3) was treated with liquid hydrogen cyanide (2 cm^3) and after 15 min, the solution was evaporated. The residue was dried azeotropically using isopropanol (4 × 100 cm^3) and then was washed with hot dry acetone to afford trimethylbenzylammonium cyanide as a white highly hygroscopic solid. This cyanide (90 mg) in dry acetone (20 cm^3) was mixed with a solution of the foregoing chloride (as **10**; 66 mg) in dry acetone (10 cm^3) and stirred at 18 °C for 40 h. The residue from evaporation was mixed with water (25 cm^3) and extracted with diethyl ether (3 × 25 cm^3), the extracted material being chromatographed on alumina with diethyl ether–light petroleum (1 : 19) to give the nitrile (as **9**) as a gum (25 mg; 8.8×10^5 disintegrations per 100 s per mmol) identical to authentic unlabelled material.

2-(4-Benzyloxy-3-methoxyphenyl)-1,3-dithiane 11

4-Benzyloxy-3-methoxybenzaldehyde (5 g, 20.7 mmol) was dissolved in chloroform (100 cm^3), propane-1,3-dithiol (2 cm^3, 2.16 g, 20 mmol) was added, the solution was saturated with hydrogen chloride and kept at room temperature overnight. The solution was washed with water (2 × 50 cm^3), 10% aqueous potassium hydroxide (2 × 50 cm^3) and water (50 cm^3), dried and evaporated. Recrystallisation of the residue from methanol–chloroform gave the *dithiane* **11** (7.2 g, 99%), mp 119.5–120 °C (Found: C, 65.2; H, 6.2; S, 19.15. $C_{18}H_{20}O_2S_2$ requires C, 65.0; H, 6.1; S, 19.3%); δ_H 2.0–2.85 (6 H, m, CH_2S and CH_2), 3.89 (3 H, s, OMe), 5.0 (1 H, s, CH), 5.11 (2 H, s, PhCH_2), 6.5–7.5 (8 H, m, ArH); *m*/*z* 332 (M^+).

4-Benzyloxy-3-methoxy[*formyl*-^{3}H]benzaldehyde 7

A solution of the dithiane **11** (2 g, 6 mmol, dried over P_2O_5) in dry DMF (50 cm^3) was warmed with stirring to 60 °C under nitrogen, sodium hydride (50% in oil, 290 mg, 6 mmol) was added and stirred at 60 °C for 5 h. (**WARNING:** *heating mixtures of sodium hydride and DMF may lead to uncontrollable exotherms.*) Tritiated water (0.6 cm^3, 33.3 mmol, 300 mCi) in DMF (1 cm^3) was added and stirred for 15 min, then unlabelled water (1.02 cm^3) was added and the cooled mixture was partitioned between chloroform and 2 M hydrochloric acid. The organic layer was washed with 2 M hydrochloric acid (6 × 50 cm^3) and the combined acidic layers were backwashed with chloroform. The combined organic solutions were evaporated and the residue recrystallised from methanol–chloroform to give the [^{3}H]dithiane (1.92 g, 96%). All this product (5.78 mmol) was dissolved in methanol–water (10 : 1, 220 cm^3) and mercuric oxide (1.25 g, 5.77 mmol) and mercuric chloride (3.38 g, 12.5 mmol) were added. After the mixture had been stirred and heated at reflux for 12 h, it was cooled, filtered and the solid washed with methanol. The filtrates were evaporated and the residue in diethyl ether (50 cm^3) was washed with half-saturated aqueous ammonium acetate (3 × 30 cm^3), brine (2 × 50 cm^3) and with water (2 × 25 cm^3). The aqueous layers were backwashed with diethyl ether and the combined ethereal solution was evaporated. The residue was purified by chromatography on silica, eluting with dichloromethane and then chloroform to give the tritiated aldehyde **7** (1.45 g, 99%), mp 62 °C; specific activity 2.83 mCi mmol^{-1}. It was identical to an authentic sample of unlabelled *O*-benzylvanillin.

2-Phenyl-4-(4-benzyloxy-3-methoxy[α-^{3}H]benzylidene)-4,5-dihydrooxazol-5-one 12

The foregoing [^{3}H]aldehyde (1 g, 4.13 mmol) was melted by heating to 100 °C and treated with acetic anhydride (3 cm^3), anhydrous sodium acetate (340 mg, 4.15 mmol) and *N*-benzoylglycine (740 mg, 4.13 mmol) to give an orange solution. Immediately a yellow crystalline solid precipitated and after 1 h the mixture was cooled and cold ethanol (50 cm^3) added. After a further 1 h, the solid was collected, washed with cold ethanol (25 cm^3) and cold diethyl ether (25 cm^3) and recrystallised from benzene to give the oxazolone **12** (980 mg, 62%), mp 197–198 °C (lit.,[21] mp for unlabelled material 196–198 °C); ν_{max}/cm^{-1} 1780, 1765, 1650; δ_H 3.96 (3 H, s, OMe), 5.2 (2 H, s, PhCH_2), 6.9 (1 H, s, CH=C), 7.2–7.6 (12 H, m, ArH), 8.16 (1 H, s, ArH).

(2*R*)-2-(4-Hydroxy-3-methoxyphenyl)[2-$^3H_1$]ethylamine 15

The synthesis was carried out through to the title compound, which was rigorously identified, with partial characterisation of the intermediates since the steps had been fully worked out on closely related systems.[8,22] The oxazolone **12** (1.1 g, 2.86 mmol) was stirred with acetone (50 cm^3), water (50 cm^3) and potassium hydroxide (500 mg, 8.93 mmol) for 30 h. The acetone was evaporated, the yellow solution was acidified and the precipitate was collected and recrystallised from ethanol–water to give 3-(4-benzyloxy-3-methoxyphenyl)-2-benzoylamino[3-^{3}H]propenoic acid **13** (1 g), mp 210 °C (lit.,[21] mp for unlabelled material,

210 °C); ν_{max}/cm^{-1} 1720, 1640. This product (1 g) in glacial acetic acid (50 cm^3) was shaken with hydrogen and 10% palladium–charcoal (200 mg) for 1 h. The catalyst was filtered off, washed with water and the filtrate was evaporated. Recrystallisation of the residue from water gave (2*R*,3*S*)- and (2*S*,3*R*)-*N*-benzoyl-3-methoxy[β-$^3H_1$]tyrosine (650 mg), mp 160–161 °C; ν_{max}(Nujol)/cm^{-1} 3300, 1720, 1640; δ_H 3.06 (2 H, m, CH_2), 3.7 (3 H, s, OMe), 4.6 (1 H, m, CH), 6.68–6.88 (3 H, m, ArH), 7.4–7.85 (5 H, m, ArH), 8.52 (1 H, d, *J* 16, NH); *m*/*z* 315 (M^+), 269, 194.

The foregoing *N*-benzoylamino acid (200 mg, 0.63 mmol) was heated at reflux in 6 M hydrochloric acid (10 cm^3) for 7 h. The solution was cooled and extracted with chloroform (3 × 10 cm^3) to remove benzoic acid. Evaporation of the aqueous solution gave (2*S*,3*R*)- and (2*R*,3*S*)-3-methoxy[β-$^3H_1$]tyrosine, **14a** and **14b** as hydrochlorides (149 mg), mp 175–178 °C which were used directly in the next step; λ_{max}/nm 284; ν_{max}/cm^{-1} 3520, 1735; δ_H (TFA) 3.4 (2 H, m, CH_2), 3.94 (3 H, s, OMe), 4.7 (1 H, m, CH), 6.6 (3 H, br s, ArH).

The foregoing amino acids **14a** and **14b** as their hydrochlorides (100 mg, 0.47 mmol) were dissolved in acetate buffer (4 cm^3, 0.2 M, pH 5.4) and the pH was readjusted to pH 5.4. After addition of tyrosine decarboxylase from *Streptococcus faecalis* (25 mg of acetone powder, Sigma) and pyridoxal phosphate (0.5 mg), the heterogenous mixture was stirred at 37 °C for 20 h. The enzyme was removed by centrifugation, the solution adjusted to pH 6.5 and then stirred with Amberlite IRC-50 (H^+) resin (2 cm^3) for 1 h. The resin and solution were transferred onto the top of a column containing Amberlite IRC-50 (H^+) (4 cm^3) which was eluted with water (175 cm^3) and the acidified eluate (hydrochloric acid) was evaporated to give the [^{3}H]ethylamine hydrochloride **15**, mp 210–212 °C (lit.,[23] 210–211 °C; lit.,[24] 212–214 °C); specific activity 2.8 mCi mmol^{-1}; λ_{max}/nm 284; ν_{max}/cm^{-1} 3400, 3100; δ_H(CD_3OD) 3.0 (4 H, m, CH_2CH_2), 3.82 (3 H, s, OMe), 6.8 (3 H, m, ArH). It was converted as earlier[1,6] into (1*RS*,4*R*)-[4-$^3H_1$]autumnaline **1a** shown to be identical to a standard unlabelled sample.[6]

Synthesis of (1*RS*,4*R*)-[4-$^3H_1$]autumnaline 1a, (1*RS*,4*S*)-[4-$^3H_1$]-autumnaline 1d and (1*RS*,4*RS*)-[4-$^3H_1$]autumnaline 1c

These three isoquinolines were synthesised from the available[5] amines **16a**, **16d** and **16c** by exactly the route developed earlier.[6] All the intermediates and the final products in the three series were shown to be identical (apart from the ^{3}H-labels) with the corresponding unlabelled authentic samples which had previously been rigorously identified. Our standard method for mixing in the (1*RS*)-[9-^{14}C]material to give the [^{3}H : ^{14}C] samples used for the incorporation experiments and to ensure constant specific activity and ^{3}H : ^{14}C ratio was as decribed in the text.

The enamine 18 derived from *O*-methylandrocymbine

The enamine **18** was prepared as previously reported[16] in 45% yield. Its ^{1}H NMR spectrum corresponded to that published except that one of the olefinic resonances was at δ 5.92 not δ 6.92 (misprint in ref. 16); *m*/*z* 383.1739 (Calc. for $C_{22}H_{25}NO_5$: M^+, 383.1733). The enamine was dissolved in tetradeuteriomethanol and aliquots were analysed by ^{1}H NMR spectroscopy after 1, 4, 8, 16 and 40 h; the signal at δ 5.03 had disappeared after 16 h and that at δ 5.92 had become a singlet. A duplicate exchange experiment gave the same results. The material from the 40 h period showed *m*/*z* 384.

Synthesis of racemic *O*-methylandrocymbine (racemic 2)

The synthesis of (1*RS*)-*O*-methylautumnaline (see its borane complex **17**) closely followed the route in the literature.[12] The yields and characteristics of the various intermediates matched those reported[12] with the final product having mp 135–137 °C (lit.,[12] mp 136–138 °C). The oxidative coupling step was based on the brief description of a related case.[11] The foregoing product in chloroform was treated with an excess of a solution of diborane in tetrahydrofuran, the solution was evaporated and the residual complex was held under vacuum to remove excess diborane. A solution of the complex (2 g) in dichloromethane (1 dm^3 distilled from phosphorus pentoxide) was treated under nitrogen in a dry box with thallium trifluoroacetate (12 g) and the mixture was stirred for 24 h. The solution was filtered, evaporated and the residue in methanol (50 cm^3) was heated under reflux for 2 h with anhydrous sodium carbonate (2 g). After filtration, the solution was evaporated and the residue in chloroform was passed through a short column of silica. The oil from evaporation of the eluate was extracted with diethyl ether and the soluble material (after treatment with charcoal if very coloured) was converted into its picrate salt in methanol. *O*-Methylandrocymbine free base was recovered by running a solution of the picrate in chloroform through a short column of alumina and was recrystallised from ethyl acetate, mp 172–174 °C (lit.,[11] mp 173–174 °C). The best yields were *ca.* 10% which, though lower than that reported[11] (20%), provided ample material for the subsequent work.

Incorporation experiments with *C. autumnale* plants

The plants, which had well formed seed capsules, were removed from outside beds to the greenhouse about 1 week before the experiments, where they were kept shaded and at outdoor temperature. They were watered once 3 days before the injections and were not watered again until 1–2 days later. Each sample of labelled autumnaline hydrochloride in aqueous solution was injected (0.02 cm^3) into each section of the seed capsule at such a concentration as to deliver 1 mg of hydrochloride per capsule; normally 20 plants were used for each experiment. The plants were allowed to metabolise for 14 days prior to harvesting.

Isolation of the alkaloids from *C. autumnale* plants

All the capsules and stalks together with the attached corms were macerated in ethanol (1 dm^3; Waring blendor); the resulting suspension was transferred to a column and kept overnight with protection against light. The plant material was eluted with ethanol (10 dm^3), the eluate evaporated and the residue was mixed with ethanol (15 cm^3), water (200 cm^3) and light petroleum (bp 60–80 °C) (250 cm^3). The mixture was filtered through Celite, the layers were separated and the aqueous layer was washed with light petroleum (bp 60–80 °C) (3 × 250 cm^3) backwashing with water (15 cm^3). The aqueous layers were extracted with chloroform (4 × 250 cm^3) and the extracts dried over anhydrous potassium carbonate and evaporated. The residue was purified by chromatography on alumina (grade I, 25 g) eluting with chloroform to obtain the alkaloid fraction. Water (80 cm^3) was equilibrated with 1 : 1 ethyl acetate–light petroleum (bp 40–60 °C) (800 cm^3) and the aqueous layer (40 cm^3) was stirred with Celite (40 g) and packed into a column. The crude alkaloids were suspended in the above equilibrated water (1 cm^3) together with enough wet 1 : 1 ethyl acetate–light petroleum (bp 40–60 °C) to form an emulsion. This was adsorbed onto Celite (2 g) and packed onto the top of the column followed by elution with wet 1 : 1 ethyl acetate–light petroleum (bp 40–60 °C) to yield a mixture of demecolcine and *O*-methylandrocymbine. Wet ethyl acetate eluted the colchicine which was recrystallised from ethyl acetate to constant specific activity and ^{3}H : ^{14}C ratio.

The fraction containing demecolcine and *O*-methylandrocymbine was mixed with synthetic *O*-methylandrocymbine prepared above (70 mg) and chromatographed on silica plates using methanol–chloroform (1 : 9) to remove fast running material. The band containing demecolcine and *O*-methylandrocymbine was rechromatographed on silica plates using 10% diethylamine in diethyl ether to separate the two alkaloids. The *O*-methylandrocymbine was converted into its picrate in methanol and recrystallised from chloroform–diethyl ether to constant specific activity and ^{3}H : ^{14}C ratio; the radioassays were carried out on the free base recovered from the picrate as above.

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