Biosynthesis. Part 29.[1] Colchicine: studies on the ring expansion step focusing on the fate of the hydrogens at C-3 of autumnaline

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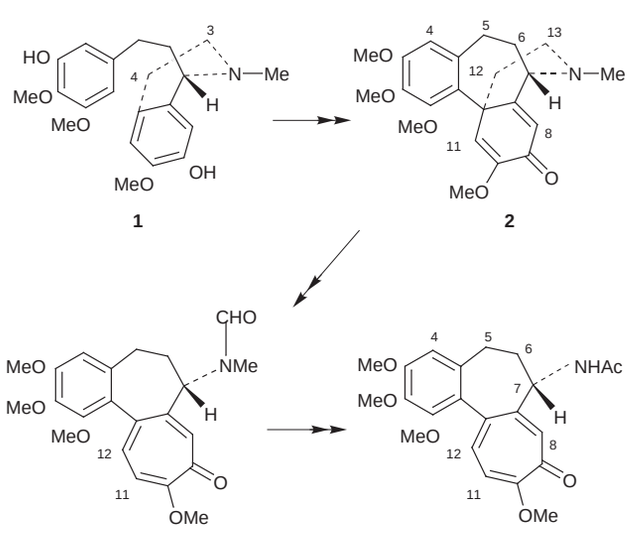
The dienone ring of the intermediate 2 undergoes expansion to form the tropolone nucleus as *N*-formyldemecolcine 3 and colchicine 4 are biosynthesised. The additional carbon atom needed to form the 7-membered ring is provided by C-12 of the dienone 2 whilst C-13 becomes the *N*-formyl group of 3. It is shown by incorporation experiments using *Colchicum* plants with precursors (as 1) stereospecifically ^{3}H-labelled at the centre corresponding to C-13 of 2 that H_S is entirely lost whereas H_R is fully retained as *N*-formyldemecolcine 3 is formed. The syntheses of the labelled precursors (as 1) are described.

Introduction

The unravelling of the biosynthetic pathway used by *Colchicum* species to build the tropolone natural product colchicine **4** led to the unexpected outcome that colchicine is a substantially modified isoquinoline alkaloid.[2–5] The overall pathway by which it is biosynthesised was set out more fully in the two preceeding papers[1,4] so Scheme 1 shows just those intermediates needed for the topic of this paper. Two or more steps are required for each of the illustrated conversions. The essential points to note are (a) that the actual isoquinoline which serves as the precursor of colchicine is (1*S*)-autumnaline **1**, (b) the dienone *O*-methylandrocymbine **2** acts as a key intermediate which later undergoes ring expansion of its dienone ring to form the tropolone nucleus of **4** with C-12 of **2** providing C-12 of colchicine and (c) C-13 of the dienone **2** is lost *en route* to **4** but is preserved in the formyl group of the minor alkaloid *N*-formyldemecolcine **3**.



Scheme 1

The ring expansion step **2** ⟶ **3** is a remarkable one of particular mechanistic interest. Yet no intermediates have been detected in *Colchicum* plants, or in their close botanical relatives, that cast any light on the steps involved. One way to get further information to help our understanding of the process is to determine the fate of the diastereotopic hydrogens of the methylene groups at C-12 and C-13 of the dienone **2**. This should be possible since, as noted above, C-12 of **2** appears at C-12 of both **3** and **4** whilst C-13 is retained in the formyl group of **3**. For obvious synthetic reasons, the plan was to introduce the tritium labels stereospecifically (or at least stereoselectively) at the corresponding sites, C-4 and C-3, of autumnaline **1**. The present paper describes the work concerning C-3 and the following one, that for C-4.

Results and discussion

Synthesis of autumnaline randomly tritiated at C-3 and its biosynthetic conversion into colchicine

Before undertaking the demanding synthesis of stereospecifically 3-^{3}H-labelled autumnalines, it was important to check whether removal of one hydrogen from C-13, the centre in **2** corresponding to C-3 of **1**, is a stereospecific process or not. This required the synthesis of (1*RS*,3*RS*)-[3-$^3H_1$]autumnaline. The C-1 racemate could be used as it had been demonstrated[4] that only the (1*S*)-autumnaline acts as a precursor of colchicine **4**. A stereospecific process acting at C-13 of the dienone **2** will eliminate 50% of the random ^{3}H-activity whereas a non-stereospecific step, because of the kinetic isotope effect, will lead to retention usually of around 80% of the ^{3}H-label.[6]

The route to the racemic (3*RS*)-[3-$^3H_1$]autumnaline **1a** is shown in Scheme 2. Tritiated nitromethane was prepared by base catalysed exchange with tritiated water and this was then condensed with 4-benzyloxy-3-methoxybenzaldehyde[7] to give the 2-(4-benzyloxy-3-methoxyphenyl)-1-nitro[1-^{3}H]ethene **5**. Lithium aluminium hydride effected complete reduction of **5** to the (1*RS*)-[1-$^3H_1$]phenethylamine **6a** which was converted into (1*RS*,3*RS*)-[3-$^3H_1$]autumnaline **1a** by the previously developed synthetic route.[3] (1*RS*)-[3-^{14}C]Autumnaline **1b**, required as internal standard for admixture with the above ^{3}H-labelled sample, was prepared essentially as previously[3] from the ^{14}C-nitrile **8** readily available from the chloride **7**. The only difference was its reduction to the amine **6b** with lithium aluminium hydride rather than by the earlier catalytic hydrogenation.[4] Again the standard sequence[3] was then used to prepare the ^{14}C-labelled sample of **1b**.

The foregoing ^{3}H- and ^{14}C-labelled samples of autumnaline as their hydrochlorides were taken together into solution in

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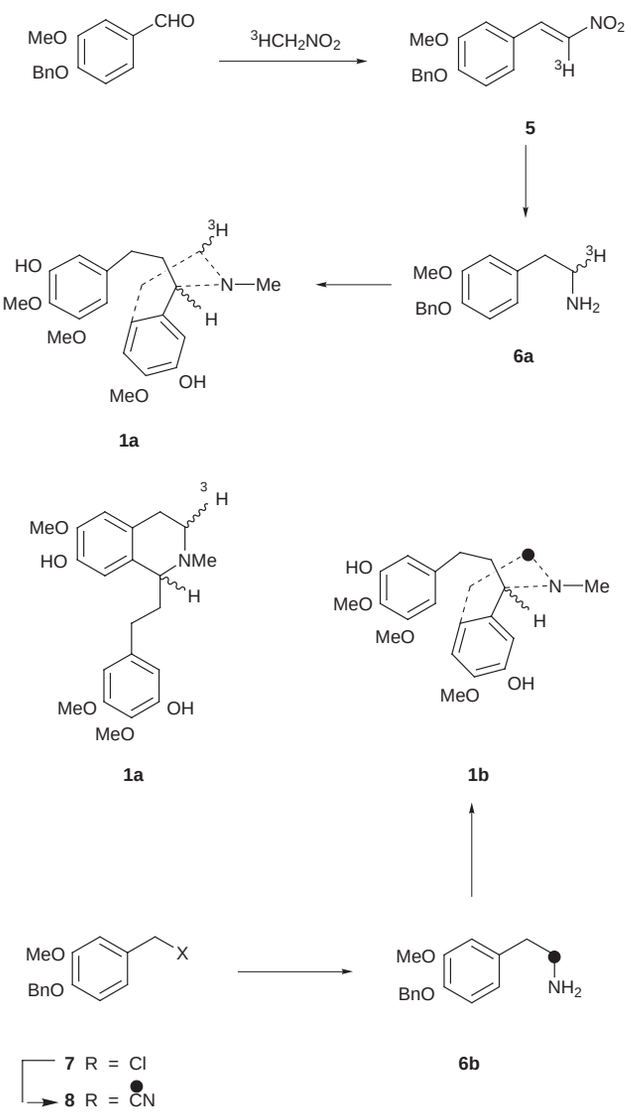


Scheme 2

amounts to give a ratio of $^3H:^{14}C$ suitable for accurate assay. The doubly labelled hydrochloride was then recrystallised several times to constant $^3H:^{14}C$ ratio. An aqueous solution of this material was injected into the seed capsules of *Colchicum speciosum* plants which were allowed to grow for two weeks before being worked-up for *N*-formyldemecolcine **3**. Since the quantity of this minor alkaloid is small, some unlabelled **3** was added at the outset of the isolation procedure to act as carrier material. This affects only the specific activity of **3** whilst leaving the $^3H:^{14}C$ ratio unchanged. The isolated **3** was recrystallised to constant specific activity and $^3H:^{14}C$ ratio before the formyl group was cleaved by acidic hydrolysis and the resultant formic acid was isolated as its crystalline *p*-bromophenacyl ester. Trials of several solvents and conditions for preparing this ester showed that the best and most reproducible yields came from using dimethylformamide (DMF). This caused a slight fall, usually about 30%, in the specific activity of the ester formed as compared with the starting *N*-formyldemecolcine **3** due to traces of formic acid in the solvent. Again this slight dilution does not affect the $^3H:^{14}C$ ratio so the method using DMF was our preferred one.

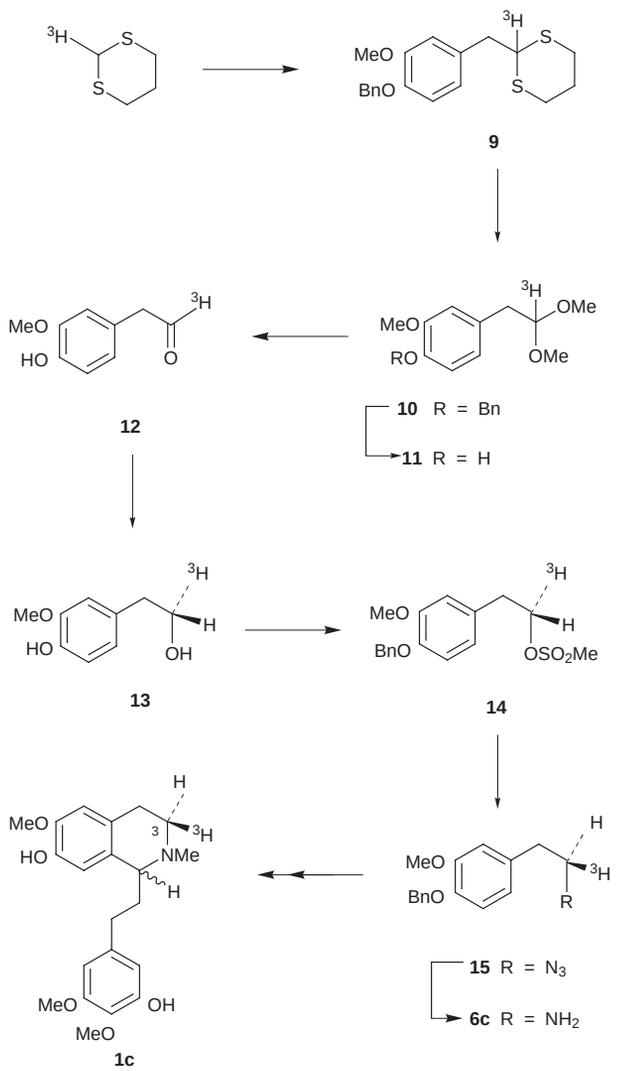
The results in Table 1 show that, well within experimental error, the $^3H:^{14}C$ ratio for *N*-formyldemecolcine **3** is 50% of that in the autumnaline administered to the plants and that the formyl group of **3** also shows the same ratio. These results are in agreement with stereospecific removal of a hydrogen from C-13 of *O*-methylandrocymbine **2** during the ring expansion process.

It was therefore worthwhile developing syntheses of stereospecifically labelled autumnalines **1**.

Syntheses of autumnaline 1 labelled stereospecifically with ^3H at C-3

Synthesis of (1*RS*,3*R*)-[3-$^3H_1$]autumnaline 1c

The key step for this synthesis was the reduction of the [*formyl*-^{3}H]aldehyde **12** with horse liver alcohol dehydrogenase, LAD (E.C. 1.1.1.1) and reduced nicotinamide adenine dinucleotide, NADH (generated *in situ*), to afford the (1*S*)-aryl[1-$^3H_1$]-ethanol **13**, Scheme 3. LAD is known[8,9] to transfer a hydride



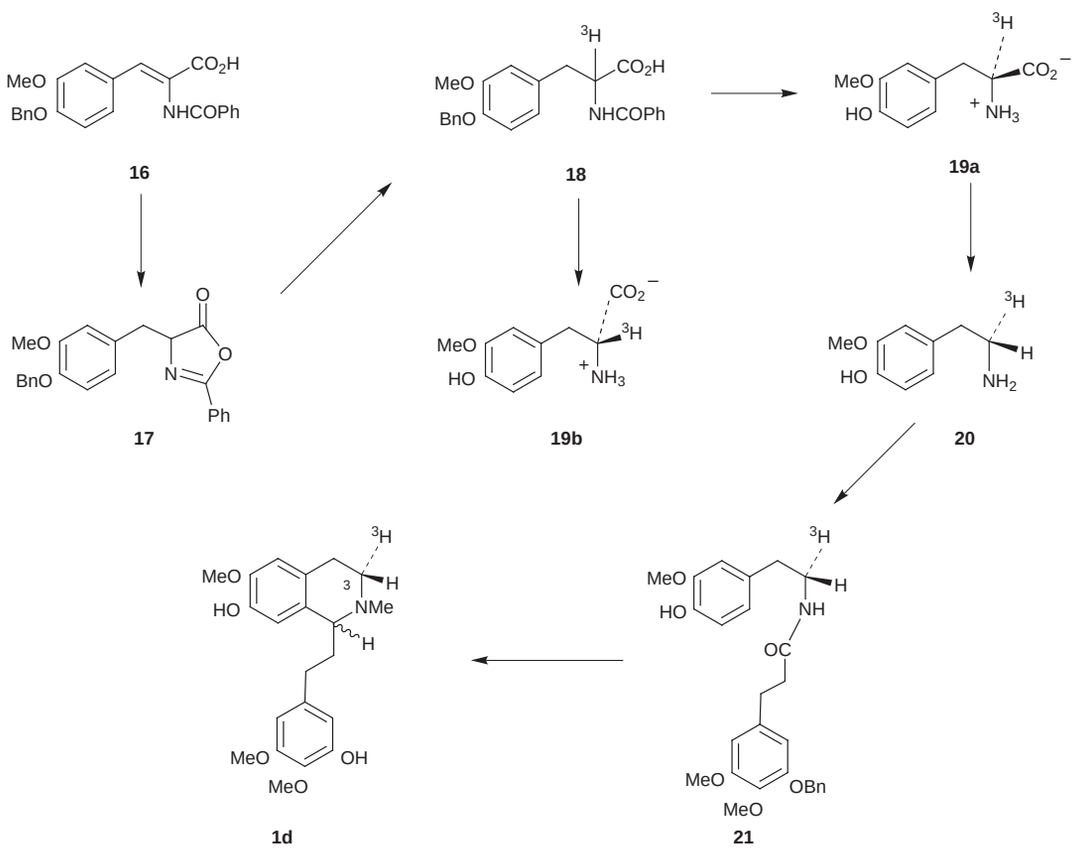
Scheme 3

equivalent to the *re*-face of an aldehyde and this stereochemical outcome has been confirmed[10–12] for many aryl aldehydes and aryl acetaldehydes similar to **12**. The later stages from **13** *via* the methanesulfonate **14** and the azide **15** through to the (1*R*)-[1-$^3H_1$]amine **6c** had previously been developed[10] and it was proved there that no significant aryl participation occurs, under the conditions used, in the azide displacement step **14** ⟶ **15**. The results obtained from that product[10] indicated that though the configurational purity of the amine **6c** was amply high enough to solve the problem, there had nevertheless been some racemisation. Accordingly in the present studies, the steps **13** ⟶ **14** and **14** ⟶ **15** were carefully monitored to keep the times of reaction to a minimum and the much weaker base, pyridine, was used for the preparation of the methanesulfonate **14**. As will be seen, these changes led to a high configurational purity being achieved.

Table 1 Incorporation of labelled autumnalines into *Colchicum* plants

	^3H : ^{14}C Ratios and ^3H-retentions (%)		
	(3*RS*)-[3-$^3H_1$,3-^{14}C] Autumnaline **1a** + **1b**	(3*R*)-[3-$^3H_1$,3-^{14}C] Autumnaline **1c** + **1b**	(3*S*)-[3-$^3H_1$,3-^{14}C] Autumnaline**1d** + **1b**
Precursor	8.9	14.4	1.92
N-Formyldemecolcine **3**	4.6	14.3	0.032
	(52)	(99)	(1.7)
p-Bromophenacyl formate	4.5	14.8	*a*
	(51)	(103)	

[a] Not determined.



Scheme 4

A method different from the earlier one[10] was used to prepare the ^3H-acetaldehyde **12**. The anion generated from 1,3-dithiane was quenched with [^3H]trifluoroacetic acid, TFA, prepared from tritiated water and TFA anhydride. Quenching with a reagent having a single protium–tritium source gives almost quantitative transfer of ^3H to the resultant dithiane; this would not be the case if tritiated water had been used as the quenching agent. Regeneration of the anion from the [2-^3H]dithiane left most of the ^3H in place because of the kinetic isotope effect and alkylation with the chloride **7** then gave the dithiane **9**. The alternative approach of preparing unlabelled **9**, generating the anion with butyllithium followed by quenching with [^3H]TFA was less satisfactory because some proton removal from the $PhCH_2O$- group competed with that from the dithiane residue. The aldehyde **12** could not be generated from the dithiane **9** without suffering extensive decomposition. However conversion first into the acetal **10** using HgO–$HgCl_2$–MeOH[13] followed by hydrogenolysis of the benzyl group and mild acidic hydrolysis of the resultant acetal **11** successfully yielded the required phenolic [*formyl*-^3H]aldehyde **12** ready for enzymic reduction. Finally, the (1*R*)-[1-$^3H_1$]amine **6c** generated by the established subsequent steps in Scheme 3 was converted by the usual sequence[3] into (1*RS*,3*R*)-[3-$^3H_1$]autumnaline **1c**.

Synthesis of (1*RS*,3*S*)-[3-$^3H_1$]autumnaline 1d

The plan was to synthesise the (2*RS*)-[2-$^3H_1$]amino acid **19a** + **19b** followed by enzymic decarboxylation specifically of the (2*S*)-enantiomer **19a** to afford the desired amine **20**, Scheme 4. The enzyme to be used was tyrosine decarboxylase which had been proved[14,15] (a) to act only on the (2*S*)-enantiomer leaving the (2*R*)-acid unchanged and (b) to carry out the decarboxylation with retention of configuration. Fortunately, this enzyme will decarboxylate substrates resembling tyrosine and trial experiments showed that the amine was formed when unlabelled **19a** + **19b** was incubated with the decarboxylase.

The starting material for the synthesis of ^3H-labelled **19a** + **19b** was the known[16] benzoylaminocinnamic acid **16**. Hydrogenation over palladium on charcoal reduced the double bond and cleaved the *O*-benzyl group which was simply replaced though with concomitant formation of some benzyl ester. This was hydrolysed by treatment with aqueous base and the resultant benzamido acid was cyclised to the oxazolone **17** by hot acetic anhydride. The introduction of the isotope then followed procedures that had been developed and monitored spectroscopically on two closely related amino acids.[12] Thus the oxazolone **17**, on treatment with deuterium oxide and pyridine, underwent rapid exchange of protium for deuterium at the chiral centre as shown by NMR spectroscopy. Slower hydrolytic

ring opening also occurred and the amide function became deuteriated but this isotope was readily washed out by dissolving the product in an excess of methanol and recovering it by evaporation. The final product was the ^{2}H analogue of **18**. The required tritiated sample **18** was prepared in the same way but now using tritiated water. Hydrolysis of **18** with hot hydrochloric acid then removed both the *N*-benzoyl and *O*-benzyl groups to afford (2*RS*)-3-methoxy[2-^{3}H]tyrosine **19a** + **19b** previously prepared by various routes in unlabelled form.[17] Incubation of this racemate with tyrosine decarboxylase (E.C. 4.1.1.25) produced a readily separable mixture of unchanged **19b** and the desired amine **20** having the (1*S*)-configuration because of the stereospecificity of the enzyme outlined above. As before, this amine **20** was converted into (1*RS*,3*S*)-[3-$^{3}H_1$]autumnaline **1d** but a slight variation of the normal route[3] was used. The phenolic amine **20** was directly acylated to give the amide **21** and now the phenolic group was *O*-benzylated to yield the fully protected amide used previously[3] in the synthesis of autumnaline.

Incorporation experiments with *Colchicum autumnale* plants

Two samples of autumnaline were now available, one having the (3*R*)-[3-$^{3}H_1$]-configuration **1c**, Scheme 3, and the other being the (3*S*)-[3-$^{3}H_1$]-isomer **1d**, Scheme 4. These, as their hydrochloride salts, were each mixed with the appropriate quantity of (1*RS*)-[3-^{14}C]autumnaline hydrochloride **1b**, Scheme 2, to give convenient $^{3}H:^{14}C$ ratios. The two samples were then diluted with enough unlabelled racemic autumnaline to allow recrystallisation to constant specific activity and constant $^{3}H:^{14}C$ ratio. Aqueous solutions of these two doubly-labelled samples of autumnaline hydrochlorides were then injected into the seed capsules of *C. autumnale* plants which were allowed to metabolise the precursor for 14 days. The plants were then worked-up with addition of unlabelled *N*-formyldemecolcine **3** to facilitate the isolation of the small amount of labelled material produced by the plants. The two rigorously purified samples of radioactive **3** were assayed by scintillation counting to determine their $^{3}H:^{14}C$ ratios. The results in Table 1 show that, as *N*-formyldemecolcine **3** is biosynthesised from autumnaline **1**, the H_S atom is removed from C-3 whereas H_R is fully retained; C-3 of **1** corresponds to C-13 of the dienone **2** into which it is converted. Finally, hydrolytic degradation of **3** derived from the (3*R*)-[$^{3}H_1$]autumnaline **1c** yielded formic acid having the same $^{3}H:^{14}C$ ratio as **3**.

Conclusions

The -CH_2–CH_2- bridge (C-3 and C-4) of autumnaline **1** is unaffected as **1** is oxidatively coupled and *O*-methylated to form *O*-methylandrocymbine **2**, Scheme 5. Thus the foregoing results establish that the ring-expansion process which generates the tropolone ring of *N*-formyldemecolcine **3**, and hence[1] of colchicine **4** itself, involves the stereospecific removal of H_S from C-13 of the dienone **2**, Scheme 5; H_R is entirely retained. The following paper[18] describes the complementary study of the fate of the hydrogens at C-4 of autumnaline **1** corresponding to C-12 of *O*-methylandrocymbine **2**. The results relating to both centres will be discussed in that paper.

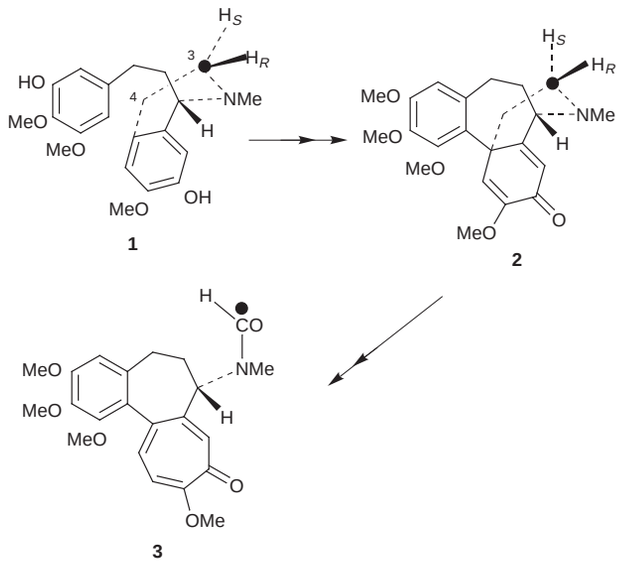
Experimental

General

For general directions see Part 30.[18]

(1*RS*)-2-(4-Benzyloxy-3-methoxyphenyl)[1-^{3}H]ethylamine 6a hydrochloride

Nitromethane (80 mg, 1.31 mmol), dimethylformamide (DMF) (1.1 cm^3), potassium hydroxide (55 mg, 0.83 mmol), dry ethanol (0.15 cm^3) and tritiated water (0.15 cm^3; 600 mCi) were stirred



Scheme 5

for 45 min, producing a yellow solution. 4-Benzyloxy-3-methoxybenzaldehyde[7] (360 mg, 1.49 mmol) in DMF (1.3 cm^3) was added, the mixture was stirred at 0 °C for 1 h then poured into 2 M hydrochloric acid (50 cm^3) containing ice. Chloroform extraction removed all the yellow product which was recovered by evaporation and purified by PLC using ethyl acetate–benzene (3:97) to yield 2-(4-benzyloxy-3-methoxyphenyl)-1-nitro[1-^{3}H]ethene **5** (60 mg; 5 mCi), mp 121–122 °C; ν_{max}/cm^{-1} 1630, 1600, 1560; δ_H 3.83 (3 H, s, MeO), 5.16 (2 H, s, CH_2), 7.0 (3 H, m, ArH), 7.3 (5 H, m, ArH), 7.7 (2 H, dd, *J* 13, CH=CH).

To a heated stirred suspension of lithium aluminium hydride (100 mg, 2.63 mmol) at reflux in diethyl ether (2 cm^3) was added a solution of the foregoing product (80 mg, 0.28 mmol) in tetrahydrofuran (THF) (5 cm^3) and heating was continued for 3 h. Aqueous 2 M sodium hydroxide was added to the cooled solution until the precipitate coagulated, and the suspension was extracted four times with diethyl ether. Evaporation of the extracts gave a gum which, in the minimum of methanol, was treated with an excess of ethereal hydrogen chloride to give [1-^{3}H]ethylamine hydrochloride **6a** (55 mg), mp 169 °C (lit.,[19] mp 168 °C), further identified by comparison with an authentic unlabelled sample.[4,19]

2-(4-Benzyloxy-3-methoxyphenyl)[1-^{14}C]ethylamine 6b hydrochloride

4-Benzyloxy-3-methoxyphenyl[1-^{14}C]acetonitrile **8** was prepared as described in Part 27.[4] The starting materials, 4-benzyloxy-3-methoxybenzyl alcohol (135 mg, 0.55 mmol) and potassium [^{14}C]cyanide (1 mCi, 1.44 mg, 0.022 mmol) afforded the crude nitrile **8** (130 mg) which was used directly below.

Dry THF (10 cm^3) was added to a mixture of lithium aluminium hydride (78 mg, 2.05 mmol) and aluminium chloride (195 mg, 1.46 mmol) and the mixture was stirred for 1.5 h. A solution of the [1-^{14}C]nitrile **8** (130 mg, 0.51 mmol) in THF (5 cm^3) was added and after the mixture had been stirred at room temperature for 2.5 h, aqueous 2 M sodium hydroxide was added dropwise until the precipitate coagulated. The solution and precipitate were each extracted four times with diethyl ether and the combined extracts evaporated. The residue in methanol (*ca.* 1 cm^3) was treated with ethereal hydrogen chloride until no more precipitate formed. The solid was recrystallised from methanol–diethyl ether to give the amine **6b** as its hydrochloride (97 mg; 0.85 mCi) mp 169 °C (lit.,[19] mp 168 °C); its identity was confirmed by comparison with authentic unlabelled material.[4,19]

[2-^{3}H]-1,3-Dithiane

1,3-Dithiane (126 mg, 1.05 mmol) in dry THF (5 cm^3) was stirred under nitrogen at −78 °C with *n*-butyllithium in hexane (0.75 cm^3, 1.06 mmol) then for 2 h at −23 °C. An aliquot was taken and quenched with D_2O; NMR showed that the dithiane contained at least 95% ^{2}H at C-2. ^{3}H-Trifluoroacetic acid (32 mCi) was added to the remainder which was stirred for 30 min then adjusted to pH 4 using dilute hydrochloric acid. The mixture was partitioned between water (2 cm^3) and chloroform (2 cm^3), the aqueous layer was extracted with chloroform (3 × 2 cm^3) and the combined organic layers were washed with water (3 × 5 cm^3). Evaporation of the organic layer gave [2-^{3}H]-1,3-dithiane, specific activity of 30 mCi mmol^{-1}.

2-(4-Benzyloxy-3-methoxybenzyl)[2-^{3}H]-1,3-dithiane 9

The anion of [2-^{3}H]-1,3-dithiane (240 mg, 2 mmol) was generated as above to which was added 4-benzyloxy-3-methoxybenzyl chloride **7** (1.95 mmol) in dry THF (3 cm^3) and the solution was kept at 0 °C overnight. It was then transferred into water (50 cm^3), extracted with diethyl ether (4 × 20 cm^3) and the extracts were washed with water (2 × 30 cm^3), dried (potassium carbonate) and evaporated. The residue crystallised from methanol to give the [^{3}H]dithiane **9** (370 mg), mp 104 °C (lit.,[10] mp for unlabelled material 105 °C), specific activity 20 mCi mmol^{-1} (Found for unlabelled material: C, 65.85; H, 6.21; S, 18.6. $C_{19}H_{22}O_2S_2$ requires C, 66.0; H, 6.4; S, 18.5%); δ_H 2.0 (2 H, m, CH_2), 2.8 (4 H, m, S-CH_2), 2.95 (2 H, d, *J* 8, ArCH_2), 3.82 (3 H, s, OMe), 4.15 (1 H, t, *J* 8, CH-S), 5.1 (2 H, s PhCH_2), 6.65 (3 H, m, ArH), 7.3 (5 H, m, ArH). *m*/*z* 346 (M$^+$), 258, 167.

1,1-Dimethoxy-2-(4-benzyloxy-3-methoxyphenyl)[1-^{3}H]ethane 10

The foregoing [^{3}H]dithiane **9** (700 mg, 2.02 mmol), mercuric oxide (480 mg, 2.22 mmol) and mercuric chloride (2.72 g, 10 mmol) were heated at reflux for 5 h in dry methanol (30 cm^3), stirring vigorously. The hot solution was filtered through a Celite plug which was washed with hot methanol and chloroform, and the filtrate was concentrated. Chloroform (50 cm^3) and water (50 cm^3) were added, and the mixture was stirred for 1 h and separated. The chloroform layer was washed with saturated aqueous ammonium acetate (3 × 20 cm^3), water (3 × 20 cm^3), dried (magnesium sulfate) and evaporated to a gum (330 mg). This was chromatographed on alumina eluting with chloroform to give the [1-^{3}H]acetal **10** (300 mg), specific activity 20 mCi mmol^{-1}; δ_H(for unlabelled material) 2.8 (2 H, d, *J* 6, ArCH_2), 3.28 (6 H, s, MeO), 3.82 (3 H, s, MeO), 4.48 (1 H, t, *J* 6, C*H*OMe), 5.07 (2 H, s, PhCH_2), 6.8 (3 H, m, ArH), 7.3 (5 H, m, ArH); this spectrum was identical to the one obtained earlier.[10]

(1*R*)-2-(4-Benzyloxy-3-methoxyphenyl)[1-^{3}H$_1$]ethylamine 6c

The steps from the acetal **10** through to the amine **6c** had been carried out previously[10] on isotopomers or stereoisomers of the compounds involved here. The experimental methods used in the present work were very similar and therefore they are described only briefly to show the quantities, radioactivities and yields involved. Samples from the earlier work[10] acted as standards. Some dilution with unlabelled substances was carried out to maintain suitable quantities of material for the synthesis; this caused the specific activity of the final product **6c** to be lower than that of the starting acetal **10**.

The foregoing acetal (200 mg, 0.66 mmol) in methanol (10 cm^3) was shaken for 4 h with hydrogen at 1 atm and 10% palladium on carbon (80 mg). The solution was filtered through Celite, the pad washed with hot methanol and the filtrate evaporated to give the dimethoxy[1-^{3}H]ethane **11** (150 mg). This in THF (3 cm^3) was stirred for 1 h with 2 M hydrochloric acid (3 cm^3) then the THF was evaporated and the aqueous layer extracted with chloroform (4 × 4 cm^3). The organic layer was washed with water and evaporated to yield the phenyl[1-^{3}H]acetaldehyde **12** (120 mg); ν_{max}/cm^{-1} 1723, 2825; δ_H 3.5 (2 H, d, *J* 2, CH_2), 3.76 (3 H, s, MeO), 6.7 (3 H, m, ArH), 9.66 (1 H, d, *J* 2, CHO).

To 0.1 M potassium phosphate buffer solution (pH 7.2, 84 cm^3) at 30 °C were added: NAD (18 mg), liver alcohol dehydrogenase (0.7 cm^3 of a suspension containing 10 mg cm^{-3}) and dioxane (3 cm^3, distilled from potassium hydroxide). A solution of the foregoing aldehyde **12** (100 mg, 0.60 mmol) in ethanol (95%, 3 cm^3) was added to the buffer solution and kept overnight at 30 °C. It was then extracted with diethyl ether (5 × 20 cm^3) and evaporated to give the crude (1*S*)-[1-^{3}H$_1$]ethanol (90 mg) in admixture with the starting aldehyde **12**. This mixture was dissolved in methanol (1 cm^3), benzyl chloride (0.2 cm^3, 0.22 g, 1.74 mmol) and anhydrous potassium carbonate (35 mg, 0.25 mmol) were added and the mixture was heated at reflux for 2 h. The solution was cooled, filtered and evaporated and the residue was purified by PLC on silica, eluting with methanol–chloroform (1 : 19) to give the *O*-benzyl ether of **13** (50 mg), mp 72–73 °C (lit.,[10] 79 °C); δ_H 1.5 (1 H, br s, OH), 2.7 (2 H, t, *J* 6, ArCH_2), 3.75 (2 H, t, *J* 6, CH_2OH), 3.8 (3 H, s, MeO), 5.08 (2 H, s, PhCH_2), 6.7 (3 H, m, ArH), 7.3 (5 H, m, ArH).

To a solution of the foregoing *O*-benzyl ether of **13** (100 mg, 0.39 mmol) in dry pyridine (1 cm^3) was added methanesulfonyl chloride (0.1 cm^3, 148 mg, 1.29 mmol) and the mixture was stirred for 1 h then evaporated. The residue was chromatographed on alumina (10 g) eluting with chloroform to yield the (1*S*)-[1-^{3}H$_1$] methanesulfonyl ester **14** (110 mg). This in 2 : 1 acetone–water (2 cm^3) was mixed with a solution of sodium azide (180 mg, 2.43 mmol) in water (0.5 cm^3) and heated at reflux with addition of acetone (1 cm^3) to dissolve remaining oily droplets. After 3 h, the acetone was evaporated and the aqueous layer extracted with diethyl ether (4 × 3 cm^3) to afford (1*R*)-2-(4-benzyloxy-3-methoxyphenyl)[1-^{3}H$_1$]ethyl azide **15** as a gum (105 mg), ν_{max}/cm^{-1} 2110. This was dissolved in diethyl ether (20 cm^3) and stirred with lithium aluminium hydride (100 mg, 2.63 mmol) at room temperature for 2 h. Excess reagent was decomposed using aqueous 2 M sodium hydroxide and the precipitate was extracted with boiling diethyl ether (3 × 10 cm^3). The combined ethereal solutions were concentrated and treated with ethereal hydrogen chloride to give crystalline (1*R*)-2-(4-benzyloxy-3-methoxyphenyl)[1-^{3}H$_1$]ethylamine **6c** hydrochloride (60 mg, 0.9 mCi), mp 169 °C further identified by comparison with an authentic unlabelled sample.[4,19]

(1*S*)-2-(4-Hydroxy-3-methoxyphenyl)[1-^{3}H$_1$]ethylamine 20

The synthesis of this substance made use of several intermediates that were known in the unlabelled series[16] or as isotopomers.[18] Because of this, and in order to conserve the labelled material, some products were passed directly to the next step and the final labelled precursor **20** was rigorously identified.

The *N*-benzoyl amino acid[16] **16** (1 g) in glacial acetic acid (50 cm^3) was shaken with 10% palladium on carbon (200 mg) and hydrogen for 1 h. The catalyst was filtered off and washed with hot water. Evaporation of the filtrate and washings gave 2-benzoylamino-3-(4-hydroxy-3-methoxyphenyl)propionic acid which was recrystallised from water (650 mg) mp 160–161 °C;[*cf.* 18] ν_{max}(Nujol)/cm^{-1} 3300, 1720, 1640; δ_H 3.06 (2 H, m, CH_2), 3.7 (3 H, s, MeO), 4.6 (1 H, m, CH), 5.1 (2 H, s, PhCH_2), 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 acid (500 mg, 1.59 mmol) was heated under reflux in methanol (20 cm^3) with sodium hydroxide (300 mg, 7.5 mmol) and benzyl chloride (935 mg, 7.39 mmol). After 4 h, 20% aqueous sodium hydroxide (4 cm^3) was added and the solution was heated under reflux for 5 h. The solution was stirred at room temperature overnight, the methanol evaporated and the aqueous solution was acidified with concentrated hydrochloric acid. Extraction with ethyl acetate (5 × 10 cm^3) gave 2-(*N*-benzoylamino)-3-(4-benzyloxy-3-methoxyphenyl)propionic acid (unlabelled **18**) which was recrystallised from aqueous

methanol (600 mg), mp 165–166 °C; ν_{max}/cm^{-1} 1730, 1640; δ_H([$^2H_6$]DMSO) 3.1 (2 H, m, CH_2), 3.72 (3 H, s, MeO), 4.65 (1 H, m, CH), 5.0 (2 H, s, PhCH_2), 6.9 (3 H, m, ArH), 7.3 (8 H, m, ArH), 7.85 (2 H, m, ArH), 8.63 (1 H, d, *J* 8, NH).

The *N*-benzoyl amino acid (unlabelled **18**) (50 mg, 0.12 mmol) was heated at 80 °C with acetic anhydride (1 cm^3) for 2 h and the solution was then evaporated to yield 2-phenyl-4-(4-benzyloxy-3-methoxybenzyl)-4,5-dihydrooxazol-5-one **17**; ν_{max}/cm^{-1} 1820, 1655; δ_H 3.2 (2 H, m, CH_2), 3.75 (3 H, s, MeO), 4.64 (1 H, t, CH), 5.02 (2 H, s, PhCH_2), 6.5–8.0 (13 H, m, ArH); the signal at δ 8.63 shown by unlabelled **18** (foregoing product) was completely absent, *m*/*z* 387, 227. This compound (100 mg, 0.26 mmol) in pyridine (0.5 cm^3) was treated with tritiated water (2.5 mg; specific activity 10 mCi mg^{-1}) and kept for 7 days before more tritiated water (2.5 mg) was added. After one more day, the solution was evaporated to dryness using vacuum transfer in a closed system. The residue crystallised on addition of a few drops of water, it was dissolved in methanol and evaporated again to remove exchangeable ^{3}H so yielding 2-(*N*-benzoylamino)-3-(4-benzyloxy-3-methoxyphenyl)[2-^{3}H]-propionic acid **18** (100 mg). This was shown to be identical, apart from the isotope, with the foregoing unlabelled sample. The exchange procedure had been studied first in the ^{2}H-series where it was shown by NMR spectroscopy that the conditions just described led to complete exchange of ^{2}H for ^{1}H at C-2 of the product as shown by the loss of the NMR signal at δ 4.65.

The foregoing *N*-benzoyl amino acid (100 mg, 0.63 mmol) was refluxed in 6 M hydrochloric acid (5 cm^3) for 15 h. The solution was cooled and extracted with chloroform (3 × 10 cm^3) to remove benzoic acid. Evaporation of the aqueous solution gave (*S*)- and (*R*)-3-methoxy[2-^{3}H]tyrosine **19a** and **19b** as the crystalline hydrochloride salt (74 mg, 1.3 mCi), mp 175–178 °C. λ_{max}/nm 284; ν_{max}/cm^{-1} 3520, 1735; δ_H(TFA) 3.4 (2 H, br, CH_2), 3.94 (3 H, s, MeO), 4.7 (1 H, m, CH), 6.6 (3 H, br s, ArH). These amino acids **19a** and **19b** were dissolved in 0.2 M acetate buffer (pH 5.4, 4 cm^3) and the pH re-adjusted to pH 5.4. The subsequent incubation with tyrosine decarboxylase from *Streptococcus faecalis* was carried out and worked up exactly as described for the isotopomers in Part 30.[18] The product, (1*S*)-2-(4-hydroxy-3-methoxyphenyl)[1-$^3H_1$]ethylamine **20**, was isolated as its hydrochloride mp 210–212 °C (lit.,[20] 210–211 °C, lit.,[21] 212–214 °C) (0.63 mCi). It was further identified by full spectroscopic and chromatographic comparison with an authentic standard (including radio-scanning of TLC comparisons).

(1*S*)-*N*-[3-(3-Benzyloxy-4,5-dimethoxyphenyl)propionoyl]-2-(4-benzyloxy-3-methoxyphenyl)[1-$^3H_1$]ethylamine

3-Benzyloxy-4,5-dimethoxyphenylpropionic acid (90 mg, 0.28 mmol) was heated with dry benzene and 10% of the solvent was distilled off. Two drops of DMF were added followed by oxalyl chloride (44 mg, 0.35 mmol) and the solution was stirred for 1 h. The resulting acid chloride was dried azeotropically three times with dry benzene and dissolved in dry dichloromethane (3 cm^3). This was slowly added to the foregoing (1*S*)-[1-$^3H_1$]-phenethylamine **20** as its hydrochloride (50 mg, 0.17 mmol) suspended in saturated aqueous sodium hydrogen carbonate (3 cm^3) and dichloromethane (2 cm^3) and stirred for 20 min. The two layers were separated, the aqueous layer extracted with chloroform (6 × 5 cm^3) and the combined organic layers were washed with water, dried and evaporated to give crude (1*S*)-*N*-[3-(3-benzyloxy-4,5-dimethoxyphenyl)propionoyl]-2-(4-hydroxy-3-methoxyphenyl)[1-$^3H_1$]ethylamine **21**; ν_{max}/cm^{-1} 3550, 3450, 1665, 1520.

The phenolic amide (150 mg, 0.33 mmol) in methanol (0.5 cm^3) was stirred vigorously with anhydrous potassium carbonate (70 mg) and benzyl chloride (66 mg, 0.52 mmol) and heated under reflux for 2 h. The solution was filtered and evaporated to give a colourless gum which was partitioned between chloroform (5 cm^3) and water (5 cm^3). The aqueous layer was extracted with chloroform (3 × 5 cm^3) and the combined organic layers were washed with water (3 × 10 cm^3). The dried organic solution was evaporated to yield the title [^{3}H]ethylamide, then purified by PLC, eluting with ethyl acetate, to give a crystalline product (130 mg), mp 110 °C (lit.,[3] mp 105–107 °C); ν_{max}/cm^{-1} 1660. It was identified by comparison with authentic material[3] and converted by the standard sequence[3] into (1*RS*,3*S*)-[3-$^3H_1$]autumnaline **1d** and this too was further identified by spectroscopic and chromatographic comparison with a standard sample.[3]

Isolation of alkaloids from incorporation experiments

This was carried out from eight plants as described elsewhere[1,18] with radio-inactive *N*-methyldemecolcine (100 mg) and *N*-formyldemecolcine (100 mg) being added to the initial methanolic extract. The eluting solvents and sizes of the fractions in the final chromatographic step were as earlier.[1]

N-Methyldemecolcine was purified by sublimation of the free base under high vacuum followed by formation and recrystallisation of the oxalate salt.[1] Demecolcine and *N*-formyldemecolcine were purified by recrystallisation as previously.[1] All three alkaloids were identified by comparison with standard samples.[1]

Degradation of *N*-formyldemecolcine

N-Formyldemecolcine (30 mg) was heated for 20 h at 100 °C in water (1 cm^3) containing concentrated sulfuric acid (0.2 cm^3). The yellow solution was cooled to 0 °C and water (50 cm^3) was added. The solution was steam distilled, collecting 400 cm^3 of distillate. This was basified to phenolphthalein with aqueous 0.1 M lithium hydroxide, the solution was evaporated to dryness and the solid was thoroughly dried at high vacuum. It was dissolved in dry DMF (0.5 cm^3), which had been distilled under vacuum from calcium hydride, and heated with *p*-bromophenacyl bromide (100 mg) at 90 °C for 2.5 h. The DMF was evaporated at high vacuum and the ether soluble part of the residue was purified by PLC on silica using benzene for development and dry diethyl ether for extraction to give *p*-bromophenacyl formate (16 mg); both chloroform and methanol caused some decomposition of the product when used for extraction from the silica. It was recrystallised from light petroleum, bp 60–80 °C, mp 97–98 °C (lit.,[22] mp 99 °C); ν_{max}/cm^{-1} 1730, 1700, identical to an authentic sample.

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