Phytochemistry, 1977, Vol. 16, pp. 1935–1937. Pergamon Press. Printed in England.

BIOGENESIS OF CYCLOBUXINE-D AND CYCLOVIROBUXINE-D IN *BUXUS SEMPERVIRENS*

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(*Received* 20 *April* 1977)

Key Word Index—*Buxus sempervirens*; Buxaceae; boxwood; biosynthesis; steroidal alkaloids; cyclovirobuxine-D; cyclobuxine-D; C-4 demethylation.

Abstract—Two major alkaloids from *Buxus sempervirens*, cyclovirobuxine-D and cyclobuxine-D, were found to be radioactively labelled following administration of mevalonic acid [2-^{14}C,(4*R*)-4-^{3}H$_1$] to freshly-harvested shoots. The ^{3}H:^{14}C atomic ratio of 3:4 in cyclovirobuxine-D indicated a biosynthetic pathway from cycloartenol involving 3-ketone and 20-ketone intermediates. A ^{3}H:^{14}C atomic ratio of *ca* 3:3 in cyclobuxine-D suggests that the 4α-methyl group of cycloartenol is lost in its formation, and this conforms with current theories of the sequence of C-4 demethylation of sterols.

INTRODUCTION

The alkaloids of the Buxaceae have interested investigators for several decades with regard to their medicinal properties, chemical separation and structural elucidation. Preliminary efforts to separate and purify the various alkaloids indicated the complex nature of *Buxus sempervirens* alkaloid extracts [1]. A convenient separation procedure for these alkaloids was first proposed by Brown and Kupchan [2], who fractionated the alkaloids by extraction from chloroform solution with aqueous solutions of progressively higher alkalinity, yielding the 'weak', 'moderate' and 'strong' base fractions. This last class of alkaloids was further fractionated by trituration into 'acetone-soluble' and 'acetone-insoluble' strong bases. Partition chromatography of the latter produced 3 major components, cyclobuxine-D (**1**) [3], cyclobuxamine-H [4] and cyclovirobuxine-D (**2**) [5].

The many new alkaloids which have been subsequently isolated and identified from *Buxus* species have been well-reviewed [6, 7]. With the exception of such alkaloid types as the buxidienenes [8, 9], which are $\Delta^{10(19),9(11)}$ compounds containing a 7-membered *B* ring, the *Buxus* alkaloids typically contain a 9β,19-cyclopropane ring as found in cycloartenol (**3**), which is thought to be the precursor triterpene [10].

The C-4 demethylation sequence involved in sterol production has been studied in several plant systems. It is known that the 4α-Me group of cycloartenol is derived from C-2 of mevalonic acid [11] and that after loss of the 4α-Me group, the 4β-Me group epimerises to the 4α-position during the production of a 4-monomethyl sterol [11–15]. A 3-ketone intermediate is proposed to explain the exchange of the 3α-H atom observed in sterol biosynthesis [11–15].

The purpose of the present investigation was to demonstrate the incorporation of radioactive MVA into the alkaloids of *B. sempervirens*, in order to gain some information on the manner of biosynthesis of cyclovirobuxine-D (**2**) and the unique 4-methylene steroid cyclobuxine-D (**1**).

RESULTS AND DISCUSSION

The distribution of radioactivity among the various alkaloid fractions following administration of mevalonic acid-[2-^{14}C,(4*R*)-4-^{3}H$_1$] is shown in Table 1. The 'weak bases' fraction contained most of the radioactivity. Among the 'strong bases' distribution of radioactivity between 'acetone-soluble' and 'acetone-insoluble' types was about equal. Upon TLC separation of the acetylated 'acetone-insoluble strong bases' the third band up from the origin, containing cyclobuxine-D (**1**) and cyclovirobuxine-D (**2**) triacetates, accounted for 17% of the

Table 1. Distribution of radioactivity among the alkaloid fractions of *B. sempervirens* after administration of mevalonic acid-[2-^{14}C,(4*R*)-4-^{3}H$_1$]

Substance	dpm of ^{14}C
Strong bases	3.51×10^5
Moderate bases	2.24×10^5
Weak bases	5.88×10^5
Acetone-soluble strong bases	1.64×10^5
Acetone-insoluble strong bases	1.50×10^5
Acetylated acetone-insoluble strong bases	1.21×10^5
TLC of acetylated acetone-insoluble strong bases on Si gel:	
Band 1	2.44×10^4
Band 2	1.01×10^4
Band 3	2.00×10^4
Band 4	732
Band 5	3.60×10^3
Band 6	1.34×10^3
Band 7	339
TLC of band 3 on $AgNO_3$–Si gel:	
Cyclobuxine-D triacetate	988
Cyclovirobuxine-D triacetate	6.03×10^3

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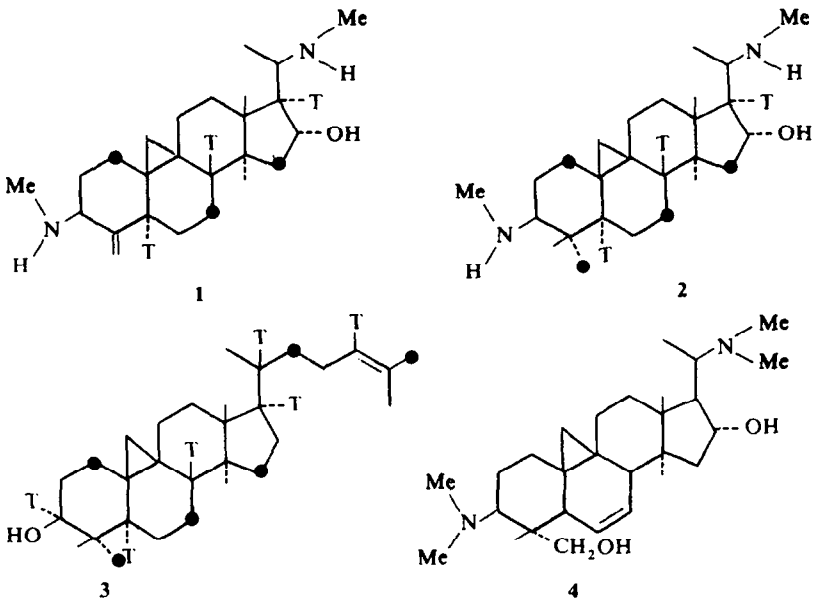

● = Carbon derived from C-2 of mevalonic acid.
T = Hydrogen derived from 4*pro-R* position of mevalonic acid.

radioactivity. After TLC separation of the components of this band, cyclobuxine-D triacetate contained 14% of the recovered radioactivity while the remainder was in cyclovirobuxine-D triacetate.

The results for the recrystallisations of squalene hexahydrochloride and cyclobuxine-D and cyclovirobuxine-D triacetates are shown in Table 2. The ^{3}H; ^{14}C atomic ratio for squalene is normally taken as 6:6. The atomic ratio for cyclovirobuxine-D was calculated on the basis of 4 ^{14}C atoms per molecule on the assumption that the *Buxus* alkaloid labelling pattern from mevalonic acid-[2-^{14}C] will be the same as in the rings of cycloartenol (3). As the alkaloids (1 and 2) are so structurally similar to cycloartenol (3), and since cyclobuxine-D, cyclovirobuxine-D and cycloartenol are all labelled from radioactive mevalonic acid in *B. sempervirens* it seemed reasonable to assume that the *Buxus* alkaloids were indeed formed from cycloartenol [10] and consequently that they had a similar ^{14}C labelling pattern. The retention of only 3 tritium atoms in cyclovirobuxine D (2) suggests that these are located at the 5α, 8β and 17α-positions as in the parent cycloartenol (3) but the tritium located at C-3(α) and C-20 of cycloartenol are lost in the biosynthesis. The formation of 3-ketone and 20-ketone intermediates followed by transamination to introduce the amino groups would result in hydrogen exchange and consequent loss of tritium at both C-3 and C-20 in *Buxus* alkaloids labelled from mevalonic acid [2-^{14}C(4*R*)-4-$^{3}H_1$]. The retention of a tritium atom at C-17 eliminates the possibility that enolisation may occur in a 20-oxo intermediate prior to transamination.

Table 2. ^{3}H:^{14}C Ratios in squalene, cyclovirobuxine-D and cyclobuxine-D from *B sempervirens* incubated with mevalonic acid-[2-^{14}C,(4*R*)-$^{3}H_1$]

Compound	Crystallisation	Specific radioactivity dpm ^{14}C/mg	^{3}H:^{14}C dpm ratio	^{3}H:^{14}C atomic ratio
Squalene hexahydrochloride	1	817	4.29	
	2	742	4.20	
	3	721	4.19	
	4	727	4.10	6:6
Cyclovirobuxine-D triacetate	1	88.4	3.35	3.19:4
	2	97.4	3.30	3.14:4
	3	88.7	3.17	3.02:4
	4	84.3	3.33	3.17:4
Cyclobuxine-D triacetate	1	14.9	4.32	3.08:3
	2	10.9	4.20	3.00:3
	3	11.7	3.46	2.47:3
	4	12.2	3.60	2.57:3

The biogenesis of cyclobuxine-D (1) can be described with less confidence since only low levels of radioactivity were incorporated thus leading to difficulties in obtaining accurate ^{3}H:^{14}C ratios. The ^{3}H:^{14}C atomic ratios obtained for the first and second crystallisations and the average value of 2.78:3 are sufficiently close to a theoretical atomic ratio of 3:3 to permit the suggestion that the 4α-Me group, labelled in cyclovirobuxine-D (2) is lost in the formation of cyclobuxine-D (1), possibly by a mechanism similar to that in cycloartenol demethylation in phytosterol biosynthesis [11] where 4α-Me group removal precedes that of the 4β-Me group. *Buxus* alkaloids such as cyclomicrophylline-A (4) with a hydroxymethylene group at C-4 have been characterised [6, 7]. The hydroxymethylene group was originally assigned the 4β-configuration but recent ^{13}C-NMR and X-ray data [16, 17] has led to the reassignment of the configuration of this group to the 4α-position. This suggests that the hydroxylation of the 4α-Me group of a 4,4-dimethyl alkaloid precursor is a first step in the oxidative removal of the 4α-Me group to give the cyclobuxines. The demethylation reaction thus follows the

same stereochemical course as in phytosterol biosynthesis but with the alkaloids it can lead to the production of a 4-methylene group from the 4β-Me group rather than its epimerisation to the 4α-configuration.

The radioactivity (Table 1), detected in the 'acetone-insoluble strong bases' fraction was comparable to that in the other alkaloid fractions. This suggests that the predominant alkaloids in the other fractions, viz., cyclobuxoxine [18–20] in the weak base fraction, cyclovirobuxeine-B [21] in the moderate bases, and cycloprotobuxine-D [22] in the acetone-soluble strong bases might be formed at a comparable rate.

EXPERIMENTAL

MS. A direct inlet system was used at 70 eV ionising energy.

Radioactive counting. Samples were dissolved in 10 ml 0.5% PPO in toluene and assayed by liquid scintillation counting with correction for quenching.

Administration of mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$]. Freshly cut shoots of *B. sempervirens* obtained in September from the University of Liverpool, Botanic Gardens, Neston, Cheshire, were incubated with mevalonic acid-[2-^{14}C,(4*R*)-4-$^{3}H_1$] (50 μCi ^{3}H; 10 μCi ^{14}C) at room temp. for 6 days. Incubations were terminated by cutting the shoots and boiling briefly in 20 ml of EtOH.

Carrier alkaloids. Cyclobuxine-D and cyclovirobuxine-D were isolated from *B. sempervirens* by the method of ref. [3].

Fractionation of material. The EtOH-boiled shoots were extracted twice for 30 min with 50 ml 0.5% HOAc in MeOH, the methanolic and ethanolic extracts pooled, and the solvents evapd. The resulting solids were partitioned between 10% HOAc and petrol (40–60°), and the aq. layer extracted a further 3x with petrol. The petrol extracts were combined and after saponification of the lipid, squalene was separated by TLC, purified via the thiourea adduct, converted to the hexahydrochloride, and recrystallised to constant sp. act.

To the aq. phase was added 30 mg of carrier 'acetone-insoluble bases', and the soln (pH 2.4) was extracted 3x with $CHCl_3$ to yield the 'weak bases'. The aq. phase was neutralised to pH 7 with 18 M NH_3 soln and similarly extracted to give the 'moderate bases'. Further NH_3 addition to pH 9.5 and extraction as before yielded the 'strong bases'. After evaporation of solvent the solids from the latter were triturated at room temp. with Me_2CO and left 18 hr at −20° The 'acetone-soluble strong bases' were removed and the remaining solids were washed twice with cold Me_2CO and dried. Acetylation was achieved by dissolving the solids in Ac_2O (3 ml) and heating at 80° for 2 hr. Excess reagent was evapd in a stream of dry N_2 and the solids dissolved in $CHCl_3$ and separated by TLC on Si gel GF_{254} (hexane–$CHCl_3$–diethylamine, 10:8:1). Material having the same R_f value (0.35) as the marker alkaloids cyclobuxine-D and cyclovirobuxine-D triacetates was eluted from the absorbent with $CHCl_3$–MeOH(1:1). After evaporation of solvent the acetylated alkaloids were taken up in $CHCl_3$. TLC on $AgNO_3$–Si gel ($CHCl_3$–MeOH, 9:1) gave two bands located by spraying with Rhodamine 6G in Me_2CO and viewing under UV. The lower band had the same R_f as cyclobuxine-D triacetate, and the upper band had the same R_f as cyclovirobuxine-D triacetate. The adsorbent was extracted with 50 ml $CHCl_3$–MeOH (1:1) and the solvents evapd down to 5 ml. To this was added 50 ml $CHCl_3$, 50 ml H_2O, 3 ml satd aq. NaCl and 1 ml 18 M NH_3 soln. The $CHCl_3$ layer was removed and concd, small portions taken for MS analysis, carrier cyclobuxine-D triacetate (25 mg) or cyclovirobuxine-D triacetate (35 mg) added as appropriate, and the compounds recrystallised from $CHCl_3$–MeOH. Cyclobuxine-D triacetate, *m/e* (rel. int.): 512 (100), 498 (6), 497 (20), 469 (5), 454 (7), 453 (21), 452 (22), 440 (17), 439 (46), 437 (11), 424 (3), 412 (5), 409 (8), 396 (11), 380 (18), 379 (32), 364 (18), 352 (19). Cyclovirobuxine-D triacetate, *m/e* (rel. int.): 528 (100), 513 (11), 485 (9), 468 (36), 455 (30), 440 (7), 425 (13), 412 (17), 395 (25), 380 (20), 369 (13), 368 (13), 356 (17), 326 (18), 322 (13), 307 (13), 253 (14).

Acknowledgements—The authors are grateful to Mr. J. K. Hulme, Director, University of Liverpool Botanic Gardens, Neston, Cheshire, for providing plant material. This work was financed by the Science Research Council.

REFERENCES

1. Schlittler, E., Heusler, K. and Freidrich, F. (1949) *Helv. Chim. Acta* **32**, 2209.
2. Brown, K. S. and Kupchan, S. M. (1962) *J. Chromatog.* **9**, 71.
3. Brown, K. S. and Kupchan, S. M. (1964) *J. Am. Chem. Soc.* **86**, 4414.
4. Brown, K. S. and Kupchan, S. M. (1964) *J. Am. Chem. Soc.* **86**, 4430.
5. Brown, K. S. and Kupchan, S. M. (1966) *Tetrahedron Letters* 2895.
6. Černý, V. and Šorm, F. (1967) in *The Alkaloids* (Manske, R. H. F. ed.) Vol. IX, p. 378. Academic Press, London.
7. Brown, K. S. (1970) in *Chemistry of the Alkaloids* (Pelletier, S. W. ed.) p. 631. Van Nostrand Reinhold, New York.
8. Khuong-Huu-Laine, F., Herlem-Gaulier, D., Khuong-Huu, O., Stanislas, E. and Goutarel, R. (1966) *Tetrahedron* **22**, 3321.
9. Tomko, J., Paverova, O., Votický, Z., Goutarel, R. and Longvaille, P. (1966) *Tetrahedron Letters*, 915.
10. Robinson, T. (1968) *The Biochemistry of the Alkaloids* p. 105. Springer-Verlag, Berlin.
11. Ghisalberti, E. L., de Souza, N. J., Rees, H. H., Goad, L. J. and Goodwin, T. W. (1969) *Chem. Commun.* 399.
12. Knapp, F. F. and Nicholas, H. J. (1969) *Phytochemistry* **8**, 207.
13. Knapp, F. F. and Nicholas, H. J. (1970) *Chem. Commun.* 399.
14. Swindell, A. C. and Gaylor, J. L. (1968) *J. Biol. Chem.* **243**, 5546.
15. Armarego, W. L. F., Goad, L. J. and Goodwin, T. W. (1973) *Phytochemistry* **12**, 2181.
16. Sangre, M., Khuong-Huu, F., Helem, D., Milliet, A., Septe, B., Beranger, G. and Lucacs, G. (1975) *Tetrahedron Letters* 1791.
17. Guilhem, J. (1975) *Tetrahedron Letters* 2937.
18. Kupchan, S. M. and Abushanab, E. (1965) *J. Org. Chem.* **30**, 3931.
19. Votický, Z., Tomko, J., Dolejs, L. and Hanus, V. (1965) *Coll. Czech. Chem. Commun.* **30**, 3705.
20. Nakano, T. and Hasegawa, M. (1965) *J. Chem. Soc.* 6688.
21. Kupchan, S. M. and Ohta, G. (1966) *J. Org. Chem.* **31**, 608.
22. Kupchan, S. M. and Kurosawa, E. (1965) *J. Org. Chem.* **30**, 2046.