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TRITERPENES AND STEROLS OF *BUXUS SEMPERVIRENS* AND LOCAL VARIATIONS IN THEIR LEVELS

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Key Word Index—*Buxus sempervirens*; Buxaceae; boxwood; sterols; triterpene monols and diols; moradiol; regional variation in composition.

Abstract—The leaves of *Buxus sempervirens* L. contain sitosterol, stigmasterol, cycloartenol, lupeol, germanicol and β-amyrin in the free state. All of these compounds, except stigmasterol, were also found in the esters fraction, as were obtusifoliol, 24-methylenelophenol, and 24-ethylidenelophenol, The triterpene diols betulin and moradiol were isolated, the latter for the first time from a plant source. In nineteen *Buxus* samples from England, Wales and Scotland, the sterol compositions were quite similar while those of the triterpene monols varied considerably.

INTRODUCTION

THE STRUCTURE of the *Buxus* alkaloids have been extensively studied during the past decade.[1] These medicinally interesting[2] compounds characteristically possess a tetracyclic triterpenoid skeleton containing a 9β,19-cyclopropane ring similar to that of cycloartenol, a precursor of plant sterols.[3]

The sterols and triterpenes of the Buxaceae, although structurally related to the alkaloids, have been much less extensively studied, and have been reported for only three members of the family. Nakano *et al.*[4] found betulinic acid, betulin and lupeol in *Buxus microphylla* var. *suffruticosa* from which they isolated the cyclomicrophyllines[5] and other alkaloids.[6] Kikuchi and Toyoda[7] found sitosterol, stigmasterol, friedelin, epifriedelanol, and pachysandiols-*A* and -*B* in *Pachysandra terminalis* from which the steroidal pachystermine alkaloids had previously been isolated.[8] Recently, Satish and Bahkuni[9] found β-amyrin and betulinic acid in *Buxus wallichiana*, the alkaloids of which had been previously studied.[10]

We accordingly decided to identify the sterols and triterpenes of *Buxus sempervirens*, notable for the number and structural variety of its alkaloids. To evaluate the differences in composition to be expected in samples of the same species taken from different locations, variations in the 4-desmethylsterols and triterpene monols were investigated in nineteen samples from England, Wales and Scotland.

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[1] CERNY, V. and SORM, F. (1967) in *The Alkaloids* (MANSKE, R. H. F., ed.), Vol. IX, p. 378, Academic Press, London.

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[6] NAKANO, T. and HASEGAWA, M. (1965) *J. Chem. Soc.* 6688.

[7] KIKUCHI, T. and TOYODA, T. (1965) *Tetrahedron Letters* 3181.

[8] KIKUCHI, T. and UYEO, S. (1965) *Tetrahedron Letters* 3473.

[9] SATISH, S. and BHAKUNI, D. S. (1972) *Phytochemistry* **11,** 2888.

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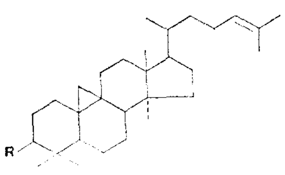
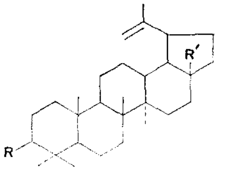
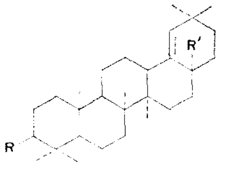
RESULTS

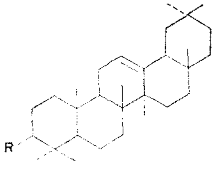
Triterpene Monols

The lipids of *Buxus sempervirens* were subjected to chromatography on alumina and the triterpene monol fractions acetylated and separated on an $AgNO_3$-alumina column. Cycloartenol (Ia) was characterised as the acetate (Ib). The low m.p. (122–123°) agreed with the accepted value.[11] Its GLC behaviour was the same as that of authentic material, and MS indicated a molecular ion at *m/e* 468 and fragmentation ions characteristic of cycloartenyl acetate.[12]


(Ia) R = OH
(Ib) R = OAc


(IIa) R = OH; R′ = Me
(IIb) R = OAc; R′ = Me
(IIc) R = OH; R′ = CH_2OH
(IId) R = OAc; R′ = CH_2OAc


(IIIa) R = OH; R′ = Me
(IIIb) R = OAc; R′ = Me
(IIIc) R = OH; R′ = CH_2OH
(IIId) R = OAc; R′ = CH_2OAc


(IVa) R = OH
(IVb) R = OAc

Lupeol (IIa) was characterized as the acetate (IIb). The m.p. (217–218°) agreed with the accepted value.[11] The IR spectrum displayed the characteristic $\Delta^{20(29)}$ absorbance at 875 and 1640 cm^{-1}.[13] The MS analysis showed peaks identical with those of authentic material, while the NMR spectrum showed resonances of the two C-29 protons at δ 4·69 (*d*, *J* 2 Hz) and 4·57 (*d*, *J* 2 Hz).

Germanicol (IIIa) was also characterized as its acetate (IIIb), after removal by crystallization of β-amyrin acetate (IVb) with which it co-chromatographed on $AgNO_3$–alumina. The high m.p. (275–276°) fell within the range of reported values.[14] The IR spectrum gave absorbances at 1025, 1250, and 1745 cm^{-1} similar to those reported.[15] The MS showed a molecular ion at *m/e* 468 and a fragment at *m/e* 204 typical of some Δ^{18} oleanenes,[16] and agreed with the spectrum of authentic material. The NMR spectrum showed the C-19 proton resonance at δ 4·88 ppm.

After hydrolysis of a sample of the germanicol-β-amyrin acetate mixture, the free alcohols were separated by $AgNO_3$–SiO_2 TLC. β-Amyrin was characterized by its GLC behaviour, m.p. (195°, lit.[11] 197°), and MS which was similar in all respects to that of the authentic material.

Sterols

GC–MS demonstrated the presence of stigmasterol and sitosterol.

Triterpene Diols

Chromatography of the lipids on alumina yielded a polar diol fraction which was purified by acetylation and column and thin layer chromatography. The diol diacetates were separated on $AgNO_3$–SiO_2 plates into two compounds. Betulin diacetate (IId) melted at 212° (lit.[11] 216°). Its identity was confirmed by MS which showed peaks at *m/e* 526 (M^+), 511 (M^+-Me), 466 (M^+-HOAc), 453 (M^+-CH_2OAc), 423 (M^+-HOAc-isopropylidene), 393

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[11] ANON (1965) *Dictionary of Organic Compounds*, Eyre & Spottiswoode, London.

[12] AUDIER, H. E., BEUGELMANS, R. and DAS, B. C. (1966) *Tetrahedron Letters* **36**, 4341.

[13] COLE, A. R. H. and THORNTON, D. W. (1957) *J. Chem. Soc.* 1332.

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(M^+-CH_2OAc–HOAc), 216, 203 and 189 (lupene skeleton fragmentation[16]). NMR measurements of the acetate agreed with published values,[17] and indicated C-28 proton resonances at δ 3·86 (*d*, *J* 11 Hz) and 4·25 ppm (*d*, *J* 11 Hz), and C-29 proton resonances at 4·59 and 4·68 ppm. IR showed the presence of the isopropylidene group[13] absorbing at 880 and 1650 cm^{-1}. After hydrolysis to betulin (IIc), MS indicated peaks at *m/e* 442 (M^+), 424 (M^+-HOH), 411 (M^+-CH_2OH), 396 (M^+-CH_2OH-Me), 340, 203 and 189.

Moradiol diacetate (IIId) melted at 275° (lit.[18] 273°). Its MS showed peaks at 526 (M^+), 466 (M^+–HOAc), 453 (M^+-CH_2OAc), 276, 216, 203 and 189 (oleanene skeleton fragmentation[16]). Comparison of the NMR spectrum of the isolated moradiol diacetate with that of authentic erythrodiol diacetate and of morolic acid acetate methyl ester[19] confirmed the identity. Peaks for the isolated moradiol diacetate were at: (δ, ppm) 0·76, 0·84, 0·90, 0·95 and 1·06 for methyl protons, 2·03 for acetate methyl protons, 3·98 (*d*, *J* 11 Hz) and 4·12 (*d*, *J* 11 Hz) for the C-28 protons, 4·50 for the 3α proton, and 5·05 for the 19β proton.

Esters

GLC of the 4-desmethylsterols derived from the ester fraction indicated sitosterol only. Analysis of the triterpene monols as the acetate derivatives on GLC showed the germanicol plus β-amyrin–cycloartenol–lupeol ratio as 3:5:2.

The 4α-methylsterols were identified by GC–MS as 24-methylenelophenol (M^+ at *m/e* 412), 24-ethylidenelophenol (M^+ at *m/e* 426), and obtusifoliol (M^+ at *m/e* 426).

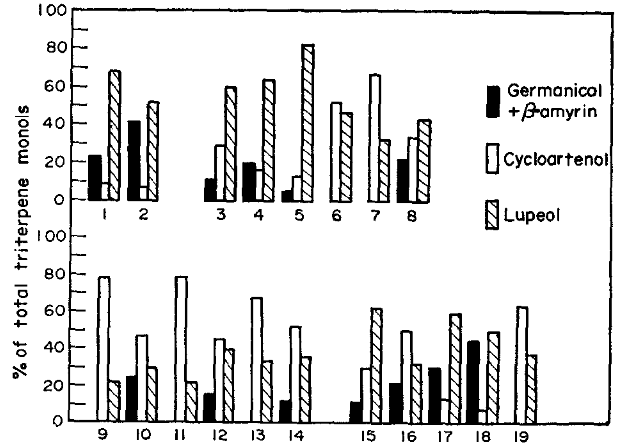

Fig. 1. Local variations in triterpene monol composition of *Buxus sempervirens*.
Samples came from: The Southwest; (1) Totnes, Devonshire, (2) Exeter, Devonshire. The Southeast; (3) Cambridge, Cambridgeshire, (4) Oxford, Oxfordshire, (5) Molesey, Surrey, (6) Englefield Green, Surrey, (7) Northiam, Sussex, (8) Woking, Surrey. Wales and the Midlands; (9) Aberystwyth, Cardiganshire, (10) Bangor, Cearnarvonshire, (11) Birmingham, Warwickshire, (12) Welshpool, Montgomeryshire, (13) Leicester, Leicestershire, (14) Swansea, Glamorganshire. North and Scotland; (15) Neston, Cheshire, (16) Kendal, Westmorland, (17) Glasgow, Lanarkshire, (18) St. Andrew's, Fifeshire, (19) Castle Douglas, Kirkcudbrightshire.

Local Variations in Triterpenes and Sterols

GLC analysis of the triterpene monols showed great variation in composition (Fig. 1.) Cycloartenol levels ranged from 7% (Exeter) to 78% (Aberystwyth), lupeol from 21%

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[17] Wilson, R. G. and Williams, D. H. (1969) *Tetrahedron* **25**, 155.

[18] Barton, D. H. R. and Brooks, C. J. W. (1951) *J. Chem. Soc.* 257.

[19] Shamma, M., Glick, R. E. and Mumma, R. O. (1962) *J. Org. Chem.* **27**, 4512.

(Birmingham) to 82% (Molesey), and germanicol and β-amyrin from nil (several samples) to 44% (St. Andrew's).

In all 19 samples studied, the sterols consisted entirely of stigmasterol and sitosterol, with the former comprising 12–14% in most cases. Exceptions occurred in the samples from Castle Douglas (19%), Leicester (18%), St. Andrew's (17%), Welshpool (16%), Exeter (6%), and Molesey (7%).

DISCUSSION

The two major triterpenes of *Buxus sempervirens*, cycloartenol and lupeol, occur in a large variety of plants.[20–22] Of the two minor triterpenes, β-amyrin is ubiquitous while germanicol and indeed all Δ^{18}-oleanenes are somewhat rare. Of the two triterpene diols, betulin occurs in several species,[14] but moradiol has never before been isolated from a plant source. The co-occurrence of lupeol and betulin and of germanicol and moradiol reveals hydroxylation of the monols at C-28 similar to that observed by Kasprzyk *et al.* for triterpene diol production in Compositae.[23,24] The apparent absence of erythrodiol, which would be formed from β-amyrin, may be due to its rapid oxidation to oleanolic acid and further hydroxylation.[23]

The 4α-methylsterols are believed to be intermediates between cycloartenol and the phytosterols.[25] According to this postulate, cycloartenol, derived from squalene-2,3-oxide, is converted to the 4-desmethylsterols through the intermediates 24-methylenecycloartenol, cycloeucalenol, obtusifoliol, 24-methylenelophenol and 24-ethylidenelophenol. The presence of cycloartenol, the phytosterols, and three of these five postulated intermediates indicates a similar biogenetic relationship in *Buxus*.

The triterpene composition of plant material may depend on such factors as age, time of collection, local climatic and soil conditions, and also on the possible occurrence of chemical races. In this study the southwest samples (1 and 2) are low in cycloartenol and comparatively high in germanicol and β-amyrin. In most of the Southeast samples (3–8), lupeol is the main triterpene; where cycloartenol predominates (samples 6 and 7) germanicol and β-amyrin are notably absent. In the Wales and Midlands samples (9–14) cycloartenol predominates in all cases. The Northern and Scottish samples (15–19) as a group have no distinctive composition although two of the Scottish samples (17 and 18) strongly resemble the Southwest samples (1 and 2) while the third (19) again shows the predominance of cycloartenol in the absence of germanicol and β-amyrin. The likelihood that workers in, say, Exeter, Molesey and Birmingham studying local plants would arrive at very different conclusions about *Buxus* triterpenes emphasizes the importance of geographical studies of this sort[26] when combined with records of plant origin (age, habitat, stage of development, mode of extraction, etc.) for possible future relevance.

EXPERIMENTAL

M.ps were determined using a Reichert hot stage. IR spectra were measured using KBr pellets. All solvents were distilled and dried before use.

Column chromatography (*CC*). Neutral alumina was de-activated to Brockmann grade III and eluted

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[20] Goad, L. J. (1967) in *Terpenoids in Plants* (Pridham, J. B., ed.), p. 159, Academic Press, London.

[21] Ourisson, G., Crabbé, P. and Rodig, O. R. (1964) *Tetracyclic Triterpenes*, Holden-Day, San Francisco.

[22] Sosa, A. and Sosa-Bourdouil, G. (1965) *Bull. Soc. Bot. Fr.* 355.

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with increasing percentages of Et_2O in light petrol. For separation of triterpene monol acetates, acid alumina impregnated with 14% (w/w) $AgNO_3$ was eluted similarly.

TLC. Kieselgel H of 500 μm thickness on 20 × 20 cm plates was used. Using $CHCl_3$, separations of triterpene diols, sterols, and triterpene monols, and separation of sterol and triterpene diol acetates were achieved. Similar layers impregnated with 10% (w/w) $AgNO_3$ were used with the following solvent systems to separate various mixtures: with C_6H_6–60/80° petrol. (1:1) for triterpene monol acetates; with $CHCl_3$–Et_2O (19:1) for germanicol and β-amyrin; with C_6H_6 for moradiol diacetate and betulin diacetate. Preparative plates were sprayed with Rhodamine 6G in acetone and examined under 254 nm light. Analytical plates were sprayed with 10% (v/v) aq. H_2SO_4 and heated at 115° for 5 min.

GLC. A Pye Series 104 instrument fitted with a FID was used with argon carrier gas at 60 ml/min. For sterols and triterpene monol acetates, a 150 cm × 4 mm column of 1% SE30 on 100–120 mesh Gas Chrom Q was run at 240° and 255° respectively. For triterpene monols a similar column of 2% OV17 was run at 273°.

MS. An AEI MS12 unit was used with a direct inlet system at 70 eV ionising energy.

GC–MS. The above GLC and MS instruments were used in conjunction. For 4α-methyl and 4-desmethylsterols a 1% JXR column with dimensions and support as above was eluted with 60 ml/min He at 255° and 240° respectively.

NMR. Spectra were recorded using $CDCl_3$ solutions with a Varian H-100D 100 MHz spectrometer at the Physico-Chemical Measurements Unit, Harwell, Didcot, Berks.

Fractionation of material. Freshly-cut *Buxus* leaves from Gloucestershire (920 g fr. wt) were extracted 2× with boiling EtOH. After solvent evaporation the residue was partitioned between petrol. and 10% (v/v) aq. HOAc. The petrol.-soluble material (10 g) was separated on alumina columns into the following fractions: esters and hydrocarbons (5·0 g) eluted with petrol., triterpene monols (2·2 g) eluted with 4–6% (v/v) Et_2O–petrol., and sterols (0·5 g) eluted with 20% (v/v) Et_2O–petrol.

Triterpene monols. The monols were acetylated and, taking several fractions with each solvent system, 600 mg were separated by argentation CC thus: germanicol and β-amyrin acetates (142 mg) eluted with 2–5% (v/v) Et_2O–petrol., cycloartenol acetate (80 mg) with 10% (v/v) Et_2O–petrol., and lupeol acetate (238 mg) with 20% (v/v) Et_2O–petrol. Acetates were re-crystallized several times from Et_2O–MeOH solutions prior to physical measurements. Germanicol and β-amyrin were separated by reduction of the acetates in Et_2O with $LiAlH_4$ and subsequent preparative TLC of the free alcohols. Germanicyl acetate. M.p. 275–276°. IR ν_{max}: 845, 1025, 1250, 1740 cm^{-1}. NMR (δ ppm): 0·74, 0·84, 0·86, 0·91, 0·94, 1·02, 1·08, 2·02, 4·50 (*m*), 4·88 (*d*, *J* 2 Hz). MS: *m/e* 468 (96%), 453 (36%), 231 (21%), 218 (46%), 205 (93%), 204 (100%), 189 (100%). Cycloartenyl acetate. M.p. 122–123°. IR: ν_{max} 1240, 1370, 1730 cm^{-1}. MS: *m/e* 468 (48%), 453 (16%), 408 (100%), 393 (46%), 365 (21%), 339 (23%), 297 (14%), 286 (47%), 271 (16%), 203 (23%). Lupeyl acetate. M.p. 217–218°. IR: ν_{max} 875, 1245, 1640, 1740 cm^{-1}. NMR (δ, ppm): 0·79, 0·84, 0·86, 0·94, 1·03, 1·68, 2·02, 4·50 (*m*), 4·57 (*d*, *J* 2 Hz), 4·69 (*d*, *J* 2 Hz). MS: *m/e* 468 (87%), 453 (15%), 408 (15%), 393 (11%), 357 (17%), 276 (20%), 249 (29%), 218 (53%), 204 (54%), 203 (45%), 189 (100%).

β-Amyrin. M.p. 195°. MS: *m/e* 426 (43%), 411 (11%), 393 (3%), 365 (3%), 218 (100%), 203 (26%), 189 (9%).

Sterols. The sterol fraction was examined by GLC and afterwards by GC–MS. Stigmasterol, MS: *m/e* 412, 394, 379, 351, 315, 300, 271, 255, 229, 213. Sitosterol, MS: *m/e* 414, 399, 396, 381, 324, 303, 273, 255, 231, 213.

Triterpene diols. A separate batch of leaves comparatively rich in diols was collected at Neston, Cheshire, in the late springtime for this purpose. The leaves (250 g fr. wt) yielded 3·9 g non-saponifiable lipid upon direct treatment with ethanolic KOH. CC gave an Et_2O-eluted fraction (524 mg) containing sterols, diols, and pigments. After acetylation, this material (260 mg) was subjected to CC and the 2% and 4% Et_2O–petrol. fractions (combined wt 65·9 mg), rich in diol diacetates, were purified by TLC to give 24·4 mg of crude diol diacetates. This mixture was separated by TLC into betulin diacetate (10·0 mg) and moradiol diacetate (8·0 mg). After crystallization from Et_2O–MeOH solutions, physical measurements were taken. The diacetates were reduced with $LiAlH_4$ to the free diols, and MS analysis were repeated. Betulin diacetate. M.p. 212°. IR: ν_{max} 880, 1245, 1650, 1750 cm^{-1}. NMR: (δ, ppm) 0·84, 0·97, 1·03, 1·68, 2·0, 3·86 (*d*, *J* 11 Hz), 4·25 (*d*, *J* 11 Hz), 4·48, 4·59, 4·68, MS: *m/e* 526 (24%), 511 (3%), 466 (100%), 453 (14%), 423 (15%), 393 (8%), 216 (19%), 203 (38%), 189 (81%). Betulin. MS: *m/e* 442 (42%), 424 (81%), 411 (42%), 396 (100%), 340 (34%), 203 (40%), 189 (34%). Moradiol diacetate. M.p. 275–276°. IR: ν_{max} 900, 980, 1015, 1025, 1245, 1750 cm^{-1}. NMR: (δ, ppm) 0·76, 0·84, 0·90, 0·95, 1·06, 2·03, 3·98 (*d*, *J* 11 Hz), 4·12 (*d*, *J* 11 Hz), 4·50 (*m*), 5·05. MS: *m/e* 526 (14%), 466 (48%), 453 (10%), 276 (16%), 216 (64%), 203 (1000%), 189 (40%). Moradiol. MS: *m/e* 442 (27%), 427 (7%), 424 (3%), 411 (100%), 393 (4%), 203 (23%), 189 (17%).

Esters. The esters and hydrocarbons fraction gave 1·4 g of non-saponifiable lipid which was fractionated by CC into triterpene monols (220 mg) eluted with 6% Et_2O–petrol. and 4α-methyl and 4-desmethylsterols (77 mg) eluted with 20% Et_2O–petrol. The triterpene monols were acetylated and analysed by TLC and GLC. The 4α-methylsterols (4·1 mg) were separated from the 4-desmethylsterols (31 mg) by TLC, examined by GLC, and the former further analysed by GC–MS. 24-Methylenelophenol, MS: *m/e* 412, 397, 394, 328, 285, 269, 267, 227. 24-Ethylidenelophenol, MS: *m/e* 426, 411, 408, 393, 328, 313, 310, 295, 285, 269, 227. Obtusifoliol, MS: *m/e* 426, 411, 408, 393, 327, 309, 245, 227.

Study of local variations in sterols and triterpene monols. From each batch of leaves collected in May 10 g were macerated in EtOH and saponified. Of the resulting non-saponifiable lipid, 30 mg were run on one TLC plate and the sterols and triterpene monols eluted and analysed by GLC. The compositions were estimated using a Vidar 6300 digital integrator.

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