Bioactive Metabolites from the South African Marine Mollusk *Trimusculus costatus*[⊥]

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A reinvestigation of extracts of the endemic South African intertidal limpet *Trimusculus costatus* yielded the known labdane diterpenes 6β,7α-diacetoxylabda-8,13*E*-dien-15-ol (**1**) and 2α,6β,7α-triacetoxylabda-8,13*E*-dien-15-ol (**2**) and three new metabolites, 6β,7α,15-triacetoxylabda-8,13*E*-diene (**3**), 3α,11-dihydroxy-9,11-seco-cholest-4,7-dien-6,9-dione (**4**), and cholest-7-en-3,5,7-triol (**5**). Chiral derivatization and X-ray analysis were used to confirm the labdane absolute configuration of **2**. Compounds **1**, **2** and **4** exhibited moderate activity (3–25 μM) against the WHCO1 human esophageal cancer cell line.

Shelled, marine pulmonate mollusks of the genus *Trimusculus* are well-adapted to the challenges of living in the intertidal zone of rocky shores and are truly amphibious, possessing a mantle cavity that has evolved to serve as both a lung and a gill.[1] Trimusculids are prolific where they occur and congregate in large colonies under shady rocky overhangs.[1] In this habitat algal growth is slow, thus reducing the significance of algae (typically exploited by other intertidal limpets) as a primary food source for trimusculids.[1–3] *Trimusculus* species have, therefore, evolved a unique feeding method and use a mucus net, secreted by the mantle glands, to filter phytoplankton out of the water column.[1] The mucus net and trapped food particles are ingested once the presence of food in the mucous net is detected by oral lobes. A secondary mucous, distinct from that used for filter feeding, is produced by numerous subepithelial glands situated in the mantle and sides of the foot.[1] This secondary mucus, containing diterpene metabolites, has chemical defense properties and has been shown to deter predation of trimusculid limpets by both seastars and fish.[2–5]

Our earlier investigation of the diterpene metabolites of the endemic South African trimusculid species *Trimusculus costatus*, the only African representative of the genus *Trimusculus*, yielded two major diterpene metabolites, 6β,7α-diacetoxylabda-8,13*E*-dien-15-ol (**1**) and 2α,6β,7α-triacetoxylabda-8,13*E*-dien-15-ol (**2**).[4] Prompted by the successful reinvestigation of other *Trimusculus* species that have afforded new minor bioactive metabolites, missed during the initial natural product studies,[6–8] we returned to extracts of *T. costatus* with the objectives of, first, isolating minor metabolites for screening against the WHCO1 esophageal cancer cell line and, second, confirming the labdane configuration of the diterpene metabolites.

Trimusculus costatus is common along the rocky shore near the coastal resort of Cintsa situated on the warm temperate southeast coast of South Africa. An acetone extract of specimens of *T. costatus* collected during the autumn of 2007 was subjected to initial polymeric reversed-phase separation followed by flash chromatography using a diol solid support. Finally, an exhaustive combination of normal-phase and diol HPLC afforded **1** and **2** and the minor metabolites 6β,7α,15-triacetoxylabda-8,13*E*-diene (**3**) 3α,11-dihydroxy-9,11-seco-cholest-4,7-dien-6,9-dione (**4**), and cholest-7-en-3,5,7-triol (**5**), from selected fractions generated from diol flash chromatography.

Diterpenes routinely dominate the natural product investigations of *Trimusculus* species and are often characterized by a 6β,7α,15-oxygenation pattern as exemplified by **1** from *T. costatus* and **6** from the Chilean species *T. peruvianus*.[4,9] Occasionally, additional oxygenation is observed at C-2, e.g., **2** from *T. costatus* and **7** from the Californian species *T. reticulatus*, or at C-3 and C-19, e.g., **8** also from *T. peruvianus*.[2,4,6] Isovalerate and acetate ester functionalities are the only two ester groups reported thus far from *Trimusculus* species and, with the exception of four Δ^{13} *Z* geometric isomers, e.g., **9** isolated from *T. peruvianus*,[8] the Δ^{13} *E* olefin is predominant. A labdane stereochemistry was randomly assigned by Manker and Faulkner to the first two metabolites, **7** and **10**, isolated from a trimusculid mollusk, and this stereochemical assignment was replicated without justification in subsequent publications describing trimusculid diterpene metabolites.[2] Recently, the application of the modified Mosher's method to **8** and a stereoselective synthesis of **10** from (+)-larixol confirmed a labdane stereochemistry in these two compounds and appeared to justify the original tenuous assignment of a labdane stereochemistry to the diterpenes isolated from *Trimusculus* species.[6,10] Confirmation that the diterpenes isolated from *T. costatus* could also be assigned to the labdane, as opposed to the *ent*-labdane series, regularly encountered in diterpenes isolated from marine mollusks,[11] was provided by esterification of **2** with (−)-camphanic chloride and crystallization of the ester product (**11**) from ethyl acetate/hexane to provide crystals suitable for X-ray analysis (Figure 1).[12] During structural elucidation of **11**, 2-fold disorder of the COO moiety of the camphanate ester was detected and modeled accordingly, resulting in refined site-occupancy factors of 0.6 and 0.4 for the major and minor components, respectively. That this disorder was not peculiar to the crystal specimen selected was subsequently verified by X-ray analysis of a second crystal specimen, which revealed the same phenomenon. The presence of this disorder did not, however, compromise the unequivocal assignment of a labdane absolute configuration to **11**, as indicated in Figure 1.

Results and Discussion

A molecular formula of $C_{26}H_{40}O_6$ was established for **3** from HRFABMS data. The congruency between the ^{1}H, ^{13}C NMR and mass data of **1**, **2**, and **3** and the presence of three ester carbonyl resonances (δ_C 169.7, 169.9, and 171.1) in the ^{13}C NMR spectrum of **3** suggested that **3** was a triacetate, isomeric with **2** and differing by one acetate unit from **1**. A three-bond gHMBC correlation from the oxymethylene protons (δ_H 4.50) in **3** to the carbonyl carbon of an acetate functionality secured the assignment of the ester moiety

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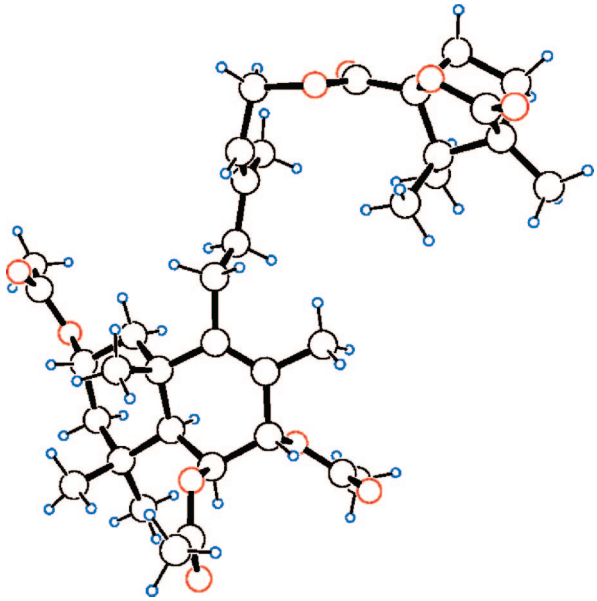
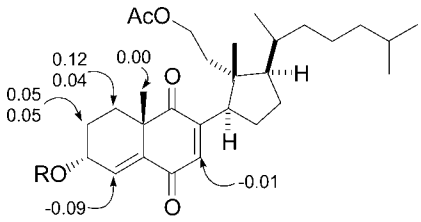


Figure 1. Perspective view of the structure of **11**. For clarity, only the major disordered component of the camphanate ester COO− group is shown (key: C black; H blue; O red).



17 R = (*R*)-MTPA
18 R = (*S*)-MTPA

Figure 2. Calculated $\Delta\delta$ values obtained from the ^{1}H NMR data (600 MHz, $CDCl_3$) of the MPTA esters (**17** and **18**) of **13**.

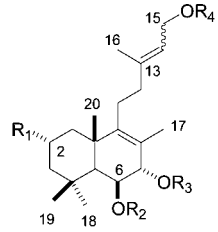
to C-15. Confirmation of the Δ^{13} *E* olefin in **3** was provided by the ^{13}C chemical shifts of the vinylic methyl (C-16) and allylic methylene (C-12) carbon resonances (δ_C 19.0 and 39.2, respectively).[13] Acetylation of a portion of **1** in the usual manner afforded **3**, which was identical in all respects with the natural product and unequivocally confirmed the structure of this compound as 6β,7α,15-triacetoxylabda-8,13*E*-diene.

The molecular formula of **4**, established as $C_{27}H_{42}O_4$ from HRFABMS data, implied seven degrees of unsaturation. The presence of two α,β-unsaturated ketones (δ_C 187.5 and 201.8) and their corresponding two trisubstituted olefins [δ_C 135.2 (d), 140.4 (s), 138.7 (d), and 153.5 (s)] and one secondary hydroxyl functionality (δ_C 63.0) was further corroborated by the IR absorption bands at 1683 and 1624 cm^{-1} and a broad absorbance band at 3420 cm^{-1}, respectively. The two α,β-unsaturated ketones accounted for four of the seven degrees of unsaturation required by the molecular formula of **4**, and the absence of any further carbonyl or olefinic functionalities suggested a tricyclic skeleton for this compound. Analysis of the ^{13}C and DEPT NMR spectra of **4** confirmed the presence of 27 carbons, comprising two trisubstituted olefins, one oxymethylene, two conjugated carbonyl, one oxymethine, two quaternary, four methyl, four methine, and eight methylene carbons. The presence of 27 carbons suggested a steroidal structure, but the tricyclic nature of **4** implied rearrangement of the typical tetracyclic steroid skeleton, such as in a 9,11-secosterol, e.g., **12**.[14] Comparison of the ^{1}H and ^{13}C NMR spectra of **4** and **12** limited the differences to the substitution pattern around the A and B rings. The position of the carbonyl at C-9 was supported by gHMBC correlations from H_3-19 (δ_H 1.37) to C-9 (δ_C 201.8). Further ^{1}H and ^{13}C NMR assignments were made from gHMBC correlations from H_3-19 to quaternary carbons C-10 (δ_C 49.2) and C-5 (δ_C 140.4), as well as to methylene carbon C-1 (δ_C 25.0). A two-bond gHMBC correlation from the olefinic methine proton H-7 (δ_H 6.69) to the remaining carbonyl carbon (δ_C 187.5), in addition to a three-bond gHMBC correlation from the olefinic proton H-4 (δ_H 6.70) to the same carbon placed the second ketone at C-6. A gCOSY correlation between H-4 and oxymethine proton H-3 (δ_H 4.34) provided the assignment of C-3 (δ_C 63.0) from gHSQC data. gCOSY correlations between oxymethine H-3 (δ_H 4.34) and H_2-2 (δ_H 1.89) facilitated the assignment of C-2 (δ_C 27.2) from gHSQC data. Two- and three-bond gHMBC correlations from H-3 to both C-2 and C-4 (δ_C 135.2) and both C-1 and C-5 confirmed the structure of ring A.

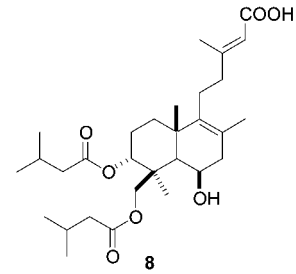
The relative configuration of the substituents around ring A of **4** was determined through NOE correlations. A correlation between H_3-19 (δ_H 1.37) and both H-1β (δ_H 1.77) and H-3 (δ_H 4.34) implied that these methyl and methine protons are situated on the same face of the ring. The configuration of the substituents around the cyclopentane ring and at C-20 was proposed from biogenetic considerations. Attempted selective acetylation of the primary alcohol at C-11 of **4** afforded both the monoacetate (**13**) and the diacetate (**14**). Subsequent application of the modified Mosher's method[15] to **13** and calculation of the $\Delta\delta$ values from the ^{1}H NMR spectra of the respective (*R*)- and (*S*)-MTPA esters (**15** and **16**) in the usual manner (Figure 2) suggested an *S* configuration at C-3 in **13**, and hence **4**.

The molecular formula of **5**, established as $C_{27}H_{46}O_3$ by HRFABMS data, implied five degrees of unsaturation. A disubstituted olefin [δ_C 130.7 (d) and 135.4 (d)] was evident from the ^{13}C and DEPT135 NMR data acquired for **5** and accounted for one of the degrees of unsaturation intimated by the molecular formula. The absence of any spectroscopic evidence to suggest the presence of other unsaturated functionalities in **5** led us to deduce a tetracyclic skeleton for this compound. A closer inspection of the ^{13}C and DEPT NMR spectra confirmed the presence of 27 carbons comprising one trisubstituted olefin, four quaternary, five methyl, six methine (one oxygenated), and 10 methylene carbons. The 27 carbons and tetracyclic nature of **5** were suggestive of a steroid nucleus. Rings C and D and the side chain of **5** were assigned through analogy with the ^{1}H and ^{13}C NMR data of **5** with those of **4**, and the individual chemical shift assignments confirmed by exhaustive gHMBC and gCOSY correlations. A two-bond gHMBC correlation from the methyl protons H_3-19 (δ_H 0.86) enabled the assignment of quaternary carbon C-10 (δ_C 36.92). Further three-bond gHMBC correlations from H_3-19 tentatively allowed us to assign methylene carbon C-1 (δ_C 34.7) and methine carbons C-9 (δ_C 51.6) and C-5 (δ_C 82.1). gHMBC correlations from the two vicinal olefinic methine protons (δ_H 6.49 and 6.22), constituting an isolated spin system, to C-5 (δ_C 82.1) and another oxygenated quaternary carbon (δ_C 79.4) placed the disubstituted olefin between these two quaternary carbons. Although the ^{1}H and ^{13}C NMR chemical shifts for C-6 and C-7 were reminiscent of epidioxy sterols (e.g., **17**),[16,17] the HRFABMS data unequivocally supported a molecular formula of $C_{27}H_{46}O_3$ for **5** (the epidioxy analogue of **5** would require $C_{27}H_{44}O_3$). In addition, a fragmentation peak corresponding to the loss of molecular oxygen, which is diagnostic for an epidioxy sterol, was absent from the mass spectrum of **5**.[18] Another three-bond gHMBC correlation from H-9 (δ_H 1.54) to δ_C 79.4 provided the assignment of C-8. A three-bond gHMBC correlation from the more deshielded of the olefinic resonances (H-7, δ6.49) to C-14 (δ_C 51.0) enabled us to assign C-7 (δ_C 130.7) and suggested that the remaining olefinic carbon resonance was C-6 (δ_C 135.4). A three-bond gHMBC correlation between H-6 and C-4 provided the assignment of C-4 (δ_C 36.89), which was further confirmed by a gCOSY correlation between H-3 (δ_H 3.95)

Chart 1



1 Δ^{13} *E*, R_1 = H, R_2 = R_3 = Ac, R_4 = H
2 Δ^{13} *E*, R_1 = OAc, R_2 = R_3 = Ac, R_4 = H
3 Δ^{13} *E*, R_1 = H, R_2 = R_3 = R_4 = Ac
6 Δ^{13} *E*, R_1 = R_3 = R_4 = H, R_2 = Ac
7 Δ^{13} *E*, R_1 = OAc, R_2 = $COCH_2CH(CH_3)_2$, R_3 = Ac, R_4 = H
9 Δ^{13} *Z*, R_1 = H, R_2 = R_3 = R_4 = Ac
10 Δ^{13} *E*, R_1 = R_4 = H R_2 = $COCH_2CH(CH_3)_2$, R_3 = Ac



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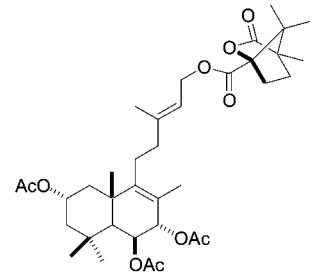

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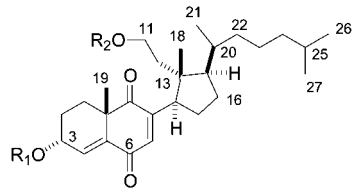
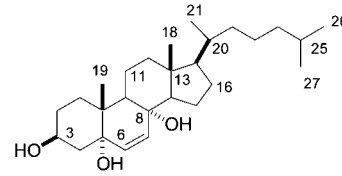


4 R_1= R_2 = H
13 R_1= H; R_2 = Ac
14 R_1 = R_2 = Ac
15 R_1= *R*-MTPA; R_2 = Ac
16 R_1= *S*-MTPA; R_2 = Ac



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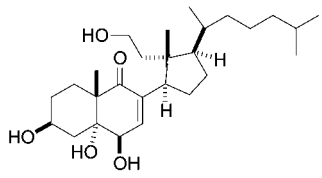

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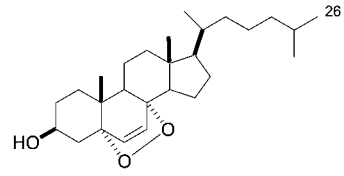

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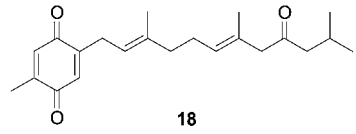

18

and both H-4β (δ_H 1.88) and H-4α (δ_H 2.09). A two-bond gHMBC correlation from H-3, as well as gCOSY correlations between H-3 and both H-2β (δ_H 1.51) and H-2α (δ_H 1.82), allowed us to assign C-2 (δ_C 30.1).

NOESY correlations between H_3-19 (δ_H 0.86) and all three H-1β (δ_H 1.67), H-2β (δ_H 1.51), and H-4β (δ_H 1.88) suggested that these protons were all on the β-face of **5**. Similarly, NOESY correlations between H-3 (δ_H 3.95) and H-1α (δ_H 1.95), H-2α (δ_H 1.82), and H-4α (δ_H 2.09) suggested a β-equatorial orientation of the hydroxyl functionality at C-3. The configurations in ring C and ring D of **5** were proposed from standard steroidal biogenetic considerations, and the configuration at C-5 was proposed from an expectation of a *trans* fusion of rings A and B. The congruency between the chemical shift data of **5** and **17** would suggest that the configuration at C-8 was consistent with that observed at this position in epidioxy sterols.

As part of an ongoing collaborative esophageal cancer research project we screened four of the metabolites (**1**−**4**) isolated from *T. costatus* for their cytotoxicity toward WHCO1 esophageal cancer cells. Globally, esophageal cancer is the eighth most common form of cancer, with remission rates rarely exceeding 10% after diagnosis.[19] In South Africa esophageal cancer is the fifth most common cancer,[20] with studies in Soweto revealing that residents there had a 5-fold higher risk of developing the cancer than the world average.[21] Although a wide range of factors have been linked to the onset of the disease, incidences of esophageal cancer in South Africa have been associated traditionally with low socioeconomic status, poor nutrition, the consumption of maize contaminated with a *Fusarium* fungus, and the use of alcohol and tobacco.[21–24]

Compounds **1**–**3** exhibited weak activity (IC_{50} 25, 24, and 84 μM, respectively) against the WHCO1 esophageal cancer cell line when compared to the commonly used chemotherapeutic agent cisplatin (IC_{50} 13 μM).[25] Compound **4**, however, exhibited reasonable activity (IC_{50} 3 μM) against this cell line. A recent study of the cellular mechanism of the antiesophageal cancer activity of the triprenylated toluquinone (**18**) and related analogues (originally isolated from the endemic South African opisthobranch mollusk *Leminda millecra*) has revealed that these compounds mediate cell death by triggering the production of reactive oxygen species (ROS), leading to the activation of signaling pathways (cJun and p38) that ultimately induce apoptosis.[26] Since ring B of **4** is structurally similar to a quinone, it is possible that this secosterol may exhibit a similar mode of action against esophageal cancer cells.

Experimental Section

General Experimental Procedures. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter calibrated at the sodium-D line (589 nm). Infrared spectra were recorded on a Perkin-Elmer Spectrum 2000 FT-IR spectrometer with compounds as films (neat) on NaCl disks. NMR spectra were acquired on Bruker 400 MHz Avance and 600 MHz Avance II spectrometers using standard pulse sequences. Chemical shifts are reported in ppm, referenced to residual solvent resonances ($CDCl_3$ δ_H 7.25, δ_C 77.2). HRFABMS data were obtained on a JEOL SX102 FAB mass spectrometer. Diaion HP-20 polystyrene divinylbenzene beads (supplied by Supelco) and Macherey-Nagel Chroma-Bond OH Diol (0.45 μm) were used for initial chromatographic separations. High-performance liquid chromatography was performed on either a Whatman's Magnum 9, Partisil 10, or a Machery-Nagel VP 250/10 Nucleosil 100-7 OH semipreparative column.

Animal Material. A collection of 1658 specimens of *Trimusculus costatus* (Krauss, 1848) was made from the densely populated colonies of this mollusk at Cintsa West, South Africa, in May 2007. The specimens were immediately placed in Me_2CO and exhaustively extracted. The Me_2CO extract was loaded onto a HP-20 column and eluted with aliquots (300 mL) of increasing concentration of Me_2CO in H_2O (0%, 40%, 60%, 80%, and 100% Me_2CO). The 60% and 80% aqueous Me_2CO fractions were each subjected to open column diol chromatography, eluting with aliquots (100 mL) of (i) EtOAc/95% hexane; (ii) EtOAc/80% hexane; (iii) EtOAc/50% hexane; and (iv) EtOAc. Normal-phase semipreparative HPLC (EtOAc/hexane, 2:3) of a portion of fraction ii of the 60% aqueous Me_2CO fraction afforded sufficient 6β,7α-diacetoxylabda-8,13*E*-dien-15-ol **1** (30 mg) and 2α,6β,7α-triacetoxylabda-8,13*E*-dien-15-ol, **2** (41 mg), for derivatization with camphanic chloride. Exhaustive semipreparative HPLC (EtOAc/hexane, 3:2) of fraction i of the 80% Me_2CO fraction afforded both 6β,7α,15-triacetoxylabda-8,13*E*-diene, **3** (3 mg, 0.002 mg/animal), and cholest-7-en-3,5,7-triol, **5** (16 mg, 0.010 mg/animal). Fraction ii of the 80% Me_2CO fraction as well as fraction iii from both the 60% and 80% Me_2CO fractions were pooled and subjected to diol semipreparative HPLC (EtOAc/hexane, 3:2) to afford 3α,11-dihydroxy-9,11-seco-cholest-4,7-dien-6,9-dione, **4** (303 mg, 0.2 mg/animal).

6β,7α-Diacetoxylabda-8,13*E*-dien-15-ol (1): pale yellow oil; $[\alpha]_D^{24}$ +93 (*c* 1.6, $CHCl_3$), lit.[4] +93; IR (film) ν_{max} 3461, 2924, 2872, 1742, 1365 cm^{-1}; ^1H NMR and ^{13}C NMR consistent with literature values;[4] HRFABMS *m/z* 406.2718 (calcd for $C_{24}H_{38}O_5$ [M^+], 406.2719).

2α,6β,7α-Triacetoxylabda-8,13*E*-dien-15-ol (2): colorless oil; $[\alpha]_D^{24}$ +60 (*c* 1.7, $CHCl_3$), lit.[4] +62; IR (film) νmax 3450, 2942, 2863, 1742, 1716 cm^{-1}; ^1H and ^{13}C NMR consistent with literature values;[4] HRFABMS *m/z* 464.2760 (calcd for $C_{26}H_{40}O_7$ [M^+], 464.2772).

6β,7α,15-Triacetoxylabda-8,13*E*-diene (3): pale yellow oil; $[\alpha]_D^{24}$ +48 (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 2957, 2871, 1737, 1679, 1622 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.37 (1H, t, J = 6.9 Hz, H-14), 5.31 (1H, br s, H-6), 4.97 (1H, br s, H-7), 4.50 (2H, d, J = 7.1 Hz, H_2-15), 2.08 (3H, s, OAc-7), 2.06 (3H, s, OAc-15), 2.03 (3H, s, OAc-6), 2.21 (2H, m, H_2-11), 2.11 (2H, m, H_2-12), 1.80 (1H, m, H-1b), 1.74 (3H, s, H_3-16), 1.69 (1H, m, H-2b), 1.61 (3H, s, H_3-17), 1.54 (1H, m, H-2a), 1.47 (1H, d, J = 1.1 Hz, H-15), 1.42 (1H, m, H-3b), 1.28 (3H, s, H_3-20), 1.21 (1H, m, H-1a), 1.19 (1H, m, H-3a), 0.97 (3H, s, H_3-18), 0.95 (3H, s, H_3-19); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 171.1 (C, OAc-7), 169.9 (C, OAc-15), 169.7 (C, OAc-6), 147.7 (C, C-9), 142.3 (C, C-13), 121.7 (C, C-8), 118.1 (CH, C-14), 73.4 (CH, C-7), 69.6 (CH, C-6), 61.3 (CH_2, C-15), 49.2 (CH, C-5), 43.1 (t, C-3), 39.5 (C, C-10), 39.2 (CH_2, C-12), 38.9 (CH_2, C-1), 33.4 (C, C-4), 33.0 (CH_3, C-18), 26.8 (CH_2, C-11), 23.1 (CH_3, C-19), 21.5 (CH_3, OAc-6), 21.2 (CH_3, OAc-15), 21.1 (CH_3, 7-OAc), 21.0 (CH_3, C-20), 19.0 (CH_2, C-2), 17.0 (CH_3, C-17), 16.6 (CH_3, C-16); HRFABMS *m/z* 449.2909 (calcd for $C_{26}H_{41}O_6$ [(M + H)$^+$], 449.2903).

3α,11-Dihydroxy-9,11-seco-cholest-4,7-dien-6,9-dione (4): bright yellow-orange oil; $[\alpha]_D$ +32 (*c* 1.5, $CHCl_3$); IR (film) ν_{max} 3420, 2957, 1683, 1624, 1462 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.70 (1H, dd, J = 4.0, 0.9 Hz, H-4), 6.69 (1H, s, H-7), 4.34 (1H, m, H-3), 3.84 (1H, ddd, J = 10.7, 10.0, 5.6 Hz, H-11b), 3.71 (1H, ddd, J = 10.7, 9.4, 5.8 Hz, H-11a), 3.54 (1H, dd, J = 11.1, 8.7 Hz, H-14), 2.17 (1H, ddd, J = 13.5, 13.4, 3.6 Hz, H-1α), 1.89 (3H, m, H_2-2, H-16b), 1.77 (1H, m, H-1β), 1.74 (1H, m, H-17), 1.71 (1H, m, H-12b), 1.68 (2H, m, H_2-15), 1.52 (1H, m, H-25), 1.50 (1H, m, H-16a), 1.43 (1H, m, H-20), 1.37 (3H, s, H_3-19), 1.35 (2H, m, H-22b, H-23b), 1.15 (2H, m, H-12a, H-23a), 1.12 (2H, m, H_2-24), 1.02 (1H, m, H-22a), 0.96 (3H, d, J = 6.7 Hz, H_3-21), 0.86 (6H, d, J = 6.6 Hz, H_3-26, H_3-27), 0.75 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 201.8 (C, C-9), 187.5 (C, C-6), 153.5 (C, C-8), 140.4 (C, C-5), 138.7 (CH, C-7), 135.2 (CH, C-4), 63.0 (CH, C-3), 59.0 (CH_2, C-11), 50.3 (CH, C-17), 49.2 (C, C-10), 47.9 (C, C-13), 44.0 (CH, C-14), 41.3 (CH_2, C-12), 39.4 (CH_2, C-24), 35.5 (CH_2, C-22), 34.6 (CH, C-20), 28.0 (CH, C-25), 27.2 (CH_2, C-2), 27.0 (CH_2, C-15), 26.8 (CH_3, C-19), 26.4 (CH_2, C-16), 25.0 (CH_2, C-1), 24.4 (CH_2, C-23), 23.0 (CH_3, C-27), 22.8 (CH_3, C-26), 18.8 (CH_3, C-21), 17.7 (CH_3, C-18); HRFABMS *m/z* 430.3082 (calcd for $C_{27}H_{42}O_4$ [M^+], 430.3083).

Cholest-7-en-3,5,7-triol (5): white, amorphous solid; $[\alpha]_D^{24}$ +4 (*c* 1.5, $CHCl_3$); IR (film) ν_{max} 3401, 2953, 1459, 1377, 1228 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 6.49 (1H, d, J = 8.5 Hz, H-7), 6.22 (1H, d, J = 8.5 Hz, H-6), 3.95 (1H, m, H-3), 2.09 (1H, ddd, J = 13.8, 5.0, 1.9 Hz, H-4α), 1.95 (1H, m, H-1α, H-12b), 1.92 (1H, m, H-16b), 1.88 (1H, dd, J = 13.8, 11.7 Hz, H-4β), 1.82 (1H, m, H-2α), 1.67 (1H, ddd, J = 13.5, 6.9, 3.5 Hz, H-1β), 1.61 (1H, m, H-11b), 1.54 (1H, dd, J = 12.2, 4.6 Hz, H-9), 1.51 (2H, m, H-2β, H-25), 1.48 (2H, m, H-14, H-15b), 1.42 (1H, m, H-11a), 1.36 (2H, m, H-16a, H-20), 1.32 (1H, m, H-22b, H-23b), 1.20 (1H, m, H-15a), 1.17 (2H, m, H-12a, H-17), 1.14 (2H, m, H-23a, H-24a), 1.01 (1H, m, H-22a), 0.88 (3H, d, J = 6.5 Hz, H_3-21), 0.86 (3H, s, H_3-19), 0.85 (3H, d, J = H_3-26), 0.84 (3H, d, J = 6.6 Hz, H_3-27), 0.78 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 135.4 (CH, C-6), 130.7 (CH, C-7), 82.1 (C, C-5), 79.4 (C, C-8), 66.4 (CH, C-3), 56.4 (CH, C-17), 51.6 (CH, C-9), 51.0 (CH, C-14), 44.7 (C, C-13), 39.4 (CH_2, C-12), 39.4 (CH_2, C-24), 36.92 (C, C-10), 36.89 (CH_2, C-4), 35.9 (CH_2, C-22), 35.2 (CH, C-20), 34.7 (CH_2, C-1), 30.1 (CH_2, C-2), 28.2 (CH_2, C-16), 28.0 (CH, C-25), 23.8 (CH_2, C-23), 23.4 (CH_2, C-15), 22.8 (CH_3, C-26), 22.5 (CH_3, C-27), 20.6 (CH_2, C-11), 18.6 (CH_3, C-21), 18.1 (CH_3, C-19), 12.6 (CH_3, C-18); HRFABMS *m/z* 419.3525 [M + H^+] (calcd for $C_{27}H_{47}O_3$, 419.3525).

Preparation of the Camphanate Ester of 2. Diterpene **2** (34 mg, 0.07 mmol), camphanic chloride (34 mg, 0.16 mmol, 2.1 equiv), Et_3N (60 μL, 7.05 mmol, 6 equiv), and DMAP (5 mg, 0.04 mmol, 0.5 equiv) were dissolved in anhydrous CH_2Cl_2 (2 mL) under an Ar atmosphere and stirred at ambient temperature (5 h). The reaction mixture was concentrated to dryness, taken up in Et_2O (5 mL), and washed with 1 M HCl (1 mL) followed by H_2O (2 × 5 mL). The organic partition was dried ($MgSO_4$) and concentrated to an amorphous solid (69 mg). Normal-phase HPLC (33% EtOAc, 67% hexane) afforded the camphanate ester **11** (38 mg, 81%) as a yellow oil. Platelets of **11**, suitable for X-ray diffraction, were grown using the slow diffusion method (hexane/EtOAc).

Camphanate ester (11): colorless plates (from hexane/EtOAc); mp 154–155 °C; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.40 (1H, dt, J = 7.1, 0.9 Hz, H-14), 5.31 (1H, m, H-6), 5.12 (1H, tt, J = 11.7, 4.1 Hz, H-2), 4.98 (1H, d, J = 0.8 Hz, H-7), 4.74 (2H, m, H_2-15), 2.42 (1H, ddd, J = 13.5, 10.8, 4.2 Hz, H-3′b), 2.19 (1H, m, H-11b), 2.13 (1H, m, H-11a), 2.10 (2H, m, H-1b and H-12b), 2.09 (1H, m, H-12a), 2.08 (3H, s, OAc-7), 2.03 (3H, s, OAc-6), 2.02 (3H, s, OAc-2), 2.02 (1H, m, H-3′a), 1.91 (1H, ddd, J = 13.2, 10.8, 4.6 Hz, H-4′b), 1.80 (1H, ddd, J = 12.3, 4.1, 1.8 Hz, H-3b), 1.76 (3H, br s, H_3-16), 1.68 (1H, ddd, J = 13.2, 9.2, 4.2 Hz, H-4′a), 1.62 (3H, s, H_3-17), 1.51 (1H, d, J = 1.2 Hz, H-5), 1.36 (3H, s, H_3-20), 1.27 (1H, dd, J = 11.9, 7.0 Hz, H-3a), 1.25 (1H, dd, J = 11.9, 7.8 Hz, H-1a), 1.10 (3H, s, H_3-8′), 1.04 (3H, s, H_3-10′), 1.03 (6H, s, H_3-19 and H_3-20), 0.94 (3H, s, H_3-9′); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 178.1 (C, C-6′), 170.5 (C, 2-OAc), 169.7 (C, 7-OAc), 169.5 (C, 6-OAc), 167.5 (C, C-1′), 146.5 (C, C-9), 143.2 (C, C-13), 122.4 (C, C-8), 117.6 (CH, C-14), 91.1 (C, C-2′), 73.2 (CH, C-7), 69.0 (CH, C-6), 68.4 (CH, C-2), 62.2 (CH_2, C-15) 54.7 (C, C-7′), 54.2 (C, C-5′), 48.8 (d, C-5), 47.8 (CH_2, C-3), 43.9 (CH_2, C-1), 40.5 (C, C-4), 39.2 (CH_2, C-12), 34.3 (C, C-10), 33.0 (CH_3, C-18), 30.6 (CH_2, C-3′), 29.0 (CH_2, C-4′), 26.7 (CH_2, C-11), 23.7 (CH_3, C-19), 22.0 (CH_3, C-20), 21.5 (CH_3, OAc-2), 21.4 (CH_3, OAc-6), 21.1 (CH_3, OAc-7), 17.0 (CH_3, C-17), 16.8 (CH_3, C-9′), 16.72 (CH_3, C-10′), 16.67 (CH_3, C-16), 9.7 (CH_3, C-8′).

Acetylation of 1. A solution of **1** (65 mg, 0.13 mmol) in pyridine (1 mL) and acetic hydride (1 mL) was stirred at ambient temperature overnight (16 h). The pyridine and acetic anhydride were removed in vacuo (0.5 mmHg) and the remaining pale yellow oil (68 mg) purified by normal-phase HPLC (50% EtOAc, 50% hexane) to afford **3** (53 mg, 90%) as a pale yellow oil: $[\alpha]_D^{24}$ +52 (*c* 4.8, $CHCl_3$), **3** from *T. costatus* $[\alpha]_D^{24}$ +48; ^1H and ^{13}C NMR data consistent with those of

the natural product; HRFABMS $[M + H]^+$ *m/z* 449.2909 (calcd for $C_{26}H_{41}O_6$, 449.2903).

Selective Acetylation of 4. The 9,11-secosterol **4** (62 mg, 0.14 mmol) and collidine (0.28 mmol, 2 equiv) were taken up in anhydrous CH_2Cl_2 (1 mL) and cooled to −78 °C under an Ar atmosphere. A solution of acetyl chloride (0.19 mmol, 2 equiv) in anhydrous CH_2Cl_2 (120 μL) was added in a dropwise fashion and stirred at −78 °C (3 h). The solution was quenched with H_2O (1 mL) and concentrated to dryness in vacuo (0.5 mmHg). The resultant yellowish solid (103 mg) was taken up in EtOAc and passed through a Teflon micropore filter to remove the whitish precipitate that formed and the yellow filtrate concentrated to a yellow oil (71 mg). The yellow oil was subjected to diol HPLC (40% EtOAc, hexane 60%) to afford both **14** (6 mg, 9%) and 3α-hydroxy-11-acetoxy-9,11-seco-cholest-4,7-dien-6,9-dione (**13**, 17 mg, 27%). Analysis of the ^{1}H NMR spectrum of each of the two fractions suggested that **14** was the diacetylated product (not further characterized) and that **13** was the desired OAc-11 product.

3α-Hydroxy-11-acetoxy-9,11-seco-cholest-4,7-dien-6,9-dione (13): yellow oil; $[\alpha]_D^{34}$ +19 (*c* 0.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.69 (1H, dd, *J* = 4.0, 0.9 Hz, H-4), 6.66 (1H, s, H-7), 4.33 (1H, m, H-3), 4.19 (1H, m, H-11b), 4.15 (1H, m, H-11a), 3.44 (1H, dd, *J* = 11.2, 8.4 Hz, H-14), 2.18 (1H, ddd, *J* = 13.5, 13.3, 3.4 Hz, H-1b), 2.01 (3H, s, OAc-11), 1.89 (1H, m, H-16b), 1.87 (2H, m, H_2-2), 1.76 (1H, m, H-1a), 1.73 (2H, m, H-12b and H-17), 1.66 (2H, m, H_2-15), 1.50 (2H, m, H-16a and H-25a), 1.43 (1H, m, H-20), 1.35 (2H, m, H-22b and H-23b), 1.35 (3H, s, H_3-19), 1.26 (1H, m, H-12a), 1.14 (1H, m, H-23a), 1.13 (2H, m, H_2-24), 1.01 (1H, m, H-22a), 0.97 (3H, d, *J* = 6.7 Hz, H_3-21), 0.85 (3H, s, H_3-27), 0.85 (3H, s, H_3-26), 0.77 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 200.4 (C, C-9), 187.5 (C, C-6), 171.0 (C, OAc-11), 153.4 (C, C-8), 140.5 (C, C-5), 138.2 (CH, C-7), 135.3 (CH, C-4), 63.1 (CH, C-3), 61.0 (CH_2, C-11), 50.7 (CH, C-17), 49.1 (C, C-10), 47.7 (C, C-13), 43.7 (CH, C-14), 39.4 (CH_2, C-24), 37.2 (CH_2, C-12), 35.4 (CH_2, C-22), 34.6 (CH, C-20), 27.9 (CH, C-25), 27.3 (CH_2, C-15), 27.2 (CH_2, C-2), 26.7 (CH_3, C-19), 26.3 (CH_2, C-16), 25.0 (CH_2, C-1), 24.4 (CH_2, C-23), 22.7 (CH_3, C-26), 22.5 (CH_3, C-27), 21.1 (CH_3, OAc-11), 18.9 (CH_3, C-21), 17.5 (CH_3, C-18); HRFABMS *m/z* 472.3188 (calcd for $C_{29}H_{44}O_5$ [M^+], 472.3189).

Preparation of the (*R*)- and (*S*)-MTPA Esters of 13. This method is representative. (*R*)-α-Methoxy-α-trifluoromethylphenylacetic acid (6 mg), DCC (48 mg), and DMAP (7 mg) were added to a solution of **13** in anhydrous CH_2Cl_2 (2 mL). The solution was shaken periodically over period of 1.5 h at ambient temperature, diluted with EtOAc (3 mL) and H_2O (1 mL), and filtered. The resulting solution was washed with 1 M HCl (1 mL), H_2O (1 mL), saturated aqueous $NaHCO_3$ (1 mL), and once more with H_2O (1 mL) in the order presented. The resulting colorless oil (7 mg) was purified by semipreparative HPLC (25% EtOAc, 75% hexane) to afford **15** (6 mg). The (*S*)-MTPA ester (**16**) was similarly prepared from **13**, using (*S*)-α-methoxy-α-trifluoromethylphenylacetic acid.

(*R*)-MTPA ester (15): yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.45 (2H, m, H-4′ and H-8′), 7.37 (3H, m, H-5′, H-6′ and H-7′), 6.68 (1H, s, H-7), 6.65 (1H, dd, *J* = 4.4, 0.9 Hz, H-4), 5.61 (1H, m, H-3), 4.20 (1H, m, H-11b), 4.16 (1H, m, H-11a), 3.49 (3H, s, H_3-9′), 3.43 (1H, dd, *J* = 11.1, 8.2 Hz, H-14), 2.01 (3H, s, OAc-11), 1.99 (2H, m, H_2-2), 1.98 (1H, m, H-1b), 1.89 (1H, m, H-16b), 1.79 (1H, m, H-1a), 1.74 (1H, m, H-17), 1.72 (1H, m, H-12b), 1.67 (2H, m, H_2-15), 1.53 (1H, m, H-16a), 1.51 (1H, m, H-25), 1.45 (1H, m, H-20), 1.37 (2H, m, H-22b and H-23b), 1.37 (3H, s, H_3-19), 1.26 (1H, m, H-12a), 1.14 (3H, m, H-23a and H_2-24), 1.01 (1H, m, H-22a), 0.98 (3H, d, *J* = 6.7 Hz, H_3-21), 0.86 (3H, d, *J* = 6.6 Hz, H_3-27), 0.86 (3H, d, *J* = 6.6 Hz, H_3-26), 0.77 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 199.7 (C, C-9), 185.6 (C, C-6), 171.0 (C, OAc-11), 165.8 (C, C-1′), 153.7 (C, C-8), 143.4 (C, C-5), 138.1 (CH, C-7), 132.5 (C, C-3′), 129.7 (CH, C-4), 129.6 (CH, C-7′), 129.6 (CH, C-5′), 128.5 (CH, C-6′), 127.1 (CH, C-8′), 127.1 (CH, C-4′), 124.1 (C, C-10′), 122.2 (C, C-2′), 67.5 (CH, C-3), 60.9 (CH_2, C-11), 55.5 (CH_3, C-9′), 50.8 (CH, C-17), 48.9 (C, C10), 47.8 (CH, C-13), 43.8 (CH, C-14), 39.4 (CH_2, C-24), 37.3 (CH_2, C-12), 35.4 (CH_2, C-22), 34.6 (CH, C-20), 28.0 (CH, C-25), 27.4 (CH_2, C-15), 26.4 (CH_3, C-19), 26.3 (CH_2, C-16), 25.2 (CH_2, C-1), 24.5 (CH_2, C-23), 24.1 (CH_2, C-2), 22.8 (CH_3, C-26), 22.5 (CH_3, C-27), 21.1 (CH_3, OAc-11), 19.0 (CH_3, C-21), 17.6 (CH_3, C-18).

(*S*)-MTPA ester (16): yellow oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.46 (2H, m, H-4′ and H-8′), 7.37 (3H, m, H-5′, H-6′ and H-7′), 6.67 (1H, s, H-7), 6.56 (1H, dd, *J* = 4.3, 0.9 Hz, H-4), 5.61 (1H, m, H-3), 4.21 (1H, m, H-11b), 4.16 (1H, m, H-11a), 3.43 (3H, s, H_3-9′), 2.10 (1H, m, H-1b), 2.04 (2H, m, H_2-2), 2.01 (3H, s, OAc-11), 1.89 (1H, m, H-16b), 1.83 (1H, H, H-1a), 1.74 (1H, m, H-12b), 1.73 (1H, m, H-17), 1.67 (2H, m, H_2-15), 1.52 (1H, m, H-16a), 1.49 (1H, m, H-25), 1.43 (1H, m, H-20), 1.37 (1H, m, H-22b), 1.36 (1H, m, H-23a), 1.24 (1H, m, H-12a), 1.17 (1H, m, H-23a), 1.14 (2H, m, H_2-24), 1.01 (1H, m, H-22a), 0.98 (3H, d, *J* = 6.7 Hz, H_3-21), 0.86 (3H, d, *J* = 6.6 Hz, H_3-27), 0.86 (3H, d, *J* = 6.6 Hz, H_3-26), 0.77 (3H, s, H_3-18); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 199.7 (C, C-9), 186.6 (C, C-6), 170.1 (C, OAc-11), 165.6 (C, C-1′), 153.6 (C, C-8), 143.2 (C, C-5), 138.1 (CH, C-7), 132.5 (C, C-3′), 129.7 (CH, C-7′), 129.7 (CH, C-7′), 129.6 (CH, C-4), 128.5 (CH, C-6′), 127.2 (CH, C-8′), 127.2 (CH, C-4′), 124.1 (C, C-10′), 122.2 (C, C-2′), 67.7 (CH, C-3), 60.9 (CH_2, C-11), 55.4 (CH_3, C-9′), 50.7 (CH, C-17), 48.9 (C, C-10), 47.8 (C, C-13), 43.8 (CH, C-14), 39.4 (CH_2, C-24), 37.3 (CH_2, C-12), 35.4 (CH_2, C-22), 34.6 (CH, C-20), 28.0 (CH, C-25), 27.4 (CH_2, C-15), 26.5 (CH_3, C-19), 26.3 (CH_2, C-16), 25.4 (CH_2, C-1), 24.4 (CH_2, C-23), 24.4 (CH_2, C-2), 22.5 (CH_3, C-27), 22.5 (CH_3, C-26), 21.1 (CH_3, 11-OAc), 19.0 (CH_3, C-21), 17.6 (CH_3, C-18).

Cell Culture. Cells were routinely maintained at 37 °C and 5% CO_2. The human esophageal cancer cell line designated as WHCO1 was derived by Professor R. Veale (University of the Witwatersrand) from South African patients with squamous cell carcinoma of the esophagus. The WHCO1 cells were maintained in DMEM, supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

MTT Assay. IC_{50} determinations were carried out using the MTT kit from Roche (cat. #1465007), according to manufacturer's instructions. Briefly, 1500 cells were seeded per well in 96-well plates. Cells were incubated (24 h), after which aqueous DMSO solutions of each compound (10 μL, with a constant final concentration of DMSO = 0.1%) were plated at various concentrations. After 48 h incubation, observations were made, and MTT (10 μL) solution added to each well. After a further 4 h incubation, solubilization solution (100 μL) was added to each well, and plates were incubated overnight. Plates were read at 595 nm on an Anthos microplate reader.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Crystal data for **11**: $C_{36}H_{52}O_{10}$, M = 644.78, colorless plate, 0.12 × 0.12 × 0.09 mm^3, monoclinic, space group $P2_1$ No. 4), a = 10.4893(2) Å, b = 7.7835(2) Å, c = 21.1003(5) Å, β = 93.518(1)°, V = 1719.46(7) Å^3, Z = 2, D_c = 1.245 g/cm^3, F_{000} = 696, Nonius Kappa CCD diffractometer, Mo Kα radiation, λ = 0.71073 Å, T = 113(2) K, $2\theta_{max}$ = 51.3°, 6480 reflections collected, 6480 unique (R_{int} = 0.0000). Final GooF = 1.049, R1 = 0.0573, wR2 = 0.1399, R indices based on 5199 reflections with $I > 2\sigma I$ (refinement on F^2), 430

parameters, 1 restraint. *Lp* corrections applied, $\mu = 0.090$ mm^{-1}. H atoms added in idealized positions in a riding model with isotropic thermal parameters 1.2–1.5× those of the parent atoms. Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC deposition number 665480). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 IEZ, UK (fax: + 44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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