Solving the Supply of Resveratrol Tetramers from Papua New Guinean Rainforest *Anisoptera* Species (Dipterocarpaceae) that Inhibit Bacterial Type III Secretion Systems

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ABSTRACT

The supply of (–)-hopeaphenol (**1**) was achieved via enzymatic biotransformation in order to provide material for pre-clinical investigation. High-throughput screening of a pre-fractionated natural product library aimed to identify compounds that inhibit the bacterial virulence type III secretion system (T3SS) identified several fractions derived from two Papua New Guinean *Anisoptera* species, showing activity against *Yersinia pseudotuberculosis* outer proteins E and H (YopE and YopH). Bioassay-directed isolation from the leaves of *A. thurifera*, and similarly *A. polyandra*, resulted in three known resveratrol tetramers, (–)-hopeaphenol (**1**), vatalbinoside A (**2**) and vaticanol B (**3**). Compounds **1**–**3** displayed IC_{50} values of 8.8, 12.5 and 9.9 μM in the reporter-gene assay, and IC_{50} values of 2.9, 4.5 and 3.3 μM in the YopH assay, respectively, which suggested that they could potentially act against the T3SS in *Yersinia*. The structures of **1-3** were confirmed through a combination of spectrometric, chemical methods and single crystal X-ray structure determinations of the natural product **1** and the permethyl ether analogue of **3**. The enzymatic hydrolysis of the β-glycoside **2** to the aglycone **1** was achieved through biotransformation using the endogenous leaf enzymes, this significantly enhanced the yield of the desired bioactive natural product from 0.08% to 1.3% and facilitates ADMET studies of (–)-hopeaphenol (**1**).

The development pipeline for new antimicrobials is poor, especially for compounds active against Gram-negative pathogens such as *Pseudomonas aeruginosa*.[1] Gram-negative bacteria are particularly problematic since their defence system consists of very efficient mechanisms to escape antibiotic pressure.[2, 3] Infections caused by *P. aeruginosa* and *Acinetobacter* spp. in particular, have increased in frequency and severity due to their capacity to generate mutator strains, persister cells and biofilms, and have therefore become progressively more difficult to treat.[1]

In the search for new antibacterial agents, numerous bacteria-specific cellular machinery or processes have been evaluated, and of interest to our research group, are compounds that disrupt the expression of bacterial effector proteins, known as exoenzymes, which are translocated *via* the type III secretion system (T3SS) from the bacteria to the infected host cell.[4, 5] These proteins alter the function of the host cell, assist the microbe to invade, resist phagocytosis, grow in deep tissues and cause disease.[6-10] The T3SS is involved in a range of pathogenic mechanisms and its activity correlates closely with disease progression and outcome.[11]

The well-studied plasmid-encoded Ysc of *Yersinia* is representative of these common virulence systems. The bacterium adheres to a eukaryotic cell and injects a set of bacterial effector proteins, Yops (*Yersinia* outer proteins), into the lumen of the target cell, and results in the inhibition of the innate immune response.[12, 13] Six different effector Yops have been identified including YopE, which inhibits phagocytosis, pore formation and cytokine production and YopH, a protein tyrosine phosphatase, which also inhibits phagocytosis, and T- and B-cell activation.[14] These Yops are essential for virulence and synthetic small molecules that inhibit Yop secretion have been identified.[15, 16] It has been shown that the salicylidene acylhydrazide class of inhibitors can attenuate virulence without affecting bacterial growth in a number of bacterial species.[17-23]

Several studies suggest that one of the key advantages of using virulence systems as targets for novel anti-infectives is a low probability for development of resistance.[4, 24] Virulence blockers target extracellular processes and are therefore less likely to be impacted by drug efflux mechanisms which have transmembrane and cytoplasmic domains.[25] It has been demonstrated that

the mechanisms of fluoroquinolone resistance and T3SS virulence are independently associated in *P. aeruginosa*.[26] Another advantage of virulence inhibitors is that cross-resistance is unlikely to evolve within or be transmutable to the beneficial microflora which do not possess T3SS machinery.[27] Lastly, since virulence blockers have a low impact on bacterial growth, selective pressure in favour of drug resistance is reduced and such mutations might result in dysfunctional virulence systems, and consequently the development of less virulent bacteria.[4, 27, 28]

To date, multiple approaches towards identifying T3SS inhibitors have been undertaken. Several studies report on the utilization of *in silico* modelling and screening campaigns with subsequent *in vitro* evaluations of target compounds. Several research groups have undertaken extensive *in vitro* screening programs of commercial, predominantly synthetic, compound libraries (ranging between 9,400 and 100,000 compounds).[15, 16, 29-32] This research has resulted in the identification of several important classes of T3SS inhibitors and notable examples include the salicylidene acylhydrazides,[15, 20] salicylanilide esters[33] thiohydrazide hydrazone derivatives[34] and N-hydroxybenzimidazoles.[35] Three smaller studies have specifically investigated T3SS inhibitors from natural sources. Vikram et al.,[36] have evaluated the T3SS inhibition of 11 citrus flavonoids whilst Tsou et al.,[37] have evaluated T3SS inhibition of 146 Traditional Chinese Medicine extracts in a *Salmonella typhimurium* model and Moir et al.,[32] have screened 1,873 plant extracts for T3SS inhibition. In addition, two articles describe activity of T3SS inhibitors *in vivo* [38, 39] and chemical optimization has resulted in more potent compounds and quantitative structure-activity relationships.

In this paper, we report the bioassay-directed isolation and *in vitro* biological activity of three compounds with activity against the T3SS components, *Y. psueudotuberculosis* YopE and YopH, from two Papua New Guinean *Anisoptera* species. Structure elucidation by a combination of NMR, MS, single crystal X-ray crystallography and chemical methods is discussed. Furthermore, enzymatic hydrolysis of one of the plant metabolites was achieved through biotransformation using the endogenous leaf enzymes, which significantly increased the yield of the desired bioactive natural product, thus enabling and expediting future ADMET and animal studies.

RESULTS AND DISCUSSION

We have undertaken a high-throughput screening (HTS) campaign of the Eskitis Institute's 'Nature Bank' natural product fraction library[40] in order to identify new compounds that potentially inhibit the T3SS system.[41] The Eskitis *Nature Bank* consists of 202,983 unique fractions derived from analytical HPLC (C_{18} monolithic) fractionation of 18,453 biota samples of plants and marine invertebrates sourced from tropical and temperate Australia, China and Papua New Guinea.[42] These samples have been extracted, pre-fractionated under optimal conditions for drug discovery,[40] and formatted for HTS.

A primary assay utilizing the bacterial clone *Y. pseudotuberculosis* YPIII(pIB102-Elux),[15] with a luminescent reporter gene under the control of the promoter for the T3SS effector protein YopE, detected three library fractions derived from both leaf extracts of two Papua New Guinean *Anisoptera* (Dipterocarpaceae) species, *A. thurifera* and *A. polyandra*, that inhibited the luminescent signal. To verify the positive hits a colorimetric assay to detect the protein tyrosine phosphatase enzyme activity of the secreted effector protein YopH was performed in conjunction with a standard antibacterial growth assay,[41] and suggested that these fractions selectively inhibited the T3SS. Bioassay-directed fractionation of the CH_2Cl_2/CH_3OH extract from *A. thurifera* and similarly *A. polyandra* resulted in the purification of three known resveratrol tetramers, (–)-hopeaphenol (**1**), vatalbinoside A (**2**) and vaticanol B (**3**).

The CH_2Cl_2/CH_3OH extract from the air-dried and ground leaves of *A. thurifera* and similarly *A. polyandra* was initially chromatographed through polyamide gel. The resulting CH_3OH eluent was then fractionated by HPLC using C_{18} bonded silica (gradient CH_3OH /H_2O/0.1% CF_3COOH). Bioassay (YopE reporter-gene assay and validation of positive hits in the YopH phosphatase assay) indicated the active fractions, and subsequent mass-directed isolation of *A. thurifera* yielded the previously reported resveratrol tetramers, (–)-hopeaphenol (**1**, 0.08% dry wt.),[43] vatalbinoside A (**2**, 1.1% dry wt.)[44] and vaticanol B (**3**, 0.25% dry wt.).[45] Mass-directed

isolation of *A. polyandra* yielded **3** (0.26% dry wt.). It is noted that the chemical composition of the leaf samples from *A. polyandra* and *A. thurifera* bear a close similarity by LC-MS.

Stilbene oligomers are biosynthesized *via* resveratrol or related phenols (*e.g*. prenylated stilbenes or aryl benzofuran derivatives) and can be classified as dimers, trimers, tetramers etc. depending on their degree of polymerization.[46] Structural characterization of these compounds is critical for inferring structure-activity information, however complete assignment of the tetrameric stilbenoids is particularly challenging since polymerization can give rise to complex structures with confusing stereochemistries and the possibility of multiple epimers, enantiomers and rotational isomers.[46, 47] In addition, further modifications such as glycosylation give rise to additional molecules with increased complexity.

In the case of **1**, the compact tetrameric structure was elucidated following 1D and 2D NMR data analysis in conjunction with comparison to literature values.[48] The absolute stereochemistry was established on the basis of optical activity ($[\alpha]_D^{25}$ -454° *c* 0.1, CH_3CH_2OH), which was in agreement with the reported value ($[\alpha]_D^{20}$ -407°, CH_3CH_2OH).[49] The structure and absolute configuration for (–)-hopeaphenol had previously been determined in 1970 by Coggon et al.[50] from X-ray crystallographic studies of the dibromodeca-*O*-methyl derivative which contained two crystallographic independent molecules, each of which are located about a two-fold symmetry axis. [A representative view of one molecule of this structure derived from the original Coggon data is provided in the supplementary tables (Supporting Information S7)]. In this present study, the high yields of **1** provided sufficient sample to carry out recrystallization experiments, which resulted in well-formed crystals of the monohydrate suitable for X-ray diffraction studies. An ORTEP representation of the structure is shown in Figure 1. The absolute configuration established for this structure was consistent with that determined for the structure of the dibromodeca-*O*-methyl derivative.

The structure and relative stereochemistry for compound **2** was assigned from its NMR spectroscopic resemblance to the aglycone **1** and in comparison to the literature values, with the H-

8b and H-8c resonances corrected as: two symmetrical and resolved broad doublet of doublets (J = 3.0, 3.0 Hz) resonating at δ_H 4.01 and 3.97 ppm respectively, compared to the previous report in which these protons were assigned as a 2H doublet of doublets (J = 10.2, 4.2 Hz).[44] Crystals of **2** were not suitable for X-ray crystallographic studies, the enantiomeric purity of **2** was confirmed on the basis of its conversion to the aglycone **1** upon acid hydrolysis (5% H_2SO_4 in CH_3CH_2OH, 65 °C, 4 h) and comparison of the optical activity of hydrolysis product ($[\alpha]_D^{20}$ -375° c =0.1, CH_3OH) to the natural product (**1**).

Compound **3** was elucidated as vaticanol B on the basis of 1D and 2D NMR spectroscopic data and in comparison to the literature.[51] Crystals of **3** could not be obtained from the nature-derived product; consequently reaction of **3** with MeI/K_2CO_3 in acetone at 40 °C for 2 h afforded the decamethyl ether **5**. Purification of the reaction mixture using C_{18} HPLC afforded **5**, (91% yield) which readily formed colourless prisms upon evaporation of the mobile phase. An ORTEP representation of the extended tetrameric rotamer vaticanol B (**5**) is shown in Figure 2.

Vatalbinoside A (**2**), vaticanol B (**3**) and (–)-hopeaphenol (**1**) exhibited IC_{50} values of 12.4, 9.9 and 8.8 μM (reporter-gene assay) and 4.5, 3.3 and 2.9 μM (YopH phosphatase assay) without detectable inhibition of bacterial growth (Table 1). These results suggest that this class of molecules targets the T3SS and may be developed as novel antibacterial agents or as chemical tools for studying the T3SS. The anti-virulence efficacy of compound (**1**) in *Y. pseudotuberculosis, P. aeruginosa* and *C. trachomatis* is discussed elsewhere.[52]

In regards to natural product drug discovery, much concern in the past and present, particularly from the pharmaceutical industry, relates to the sufficient supply of a natural product lead or drug for more detailed pharmacological evaluations, both in the *in vitro* and/or *in vivo* setting. The stilbenoid compounds from these current studies are all structurally complex and total syntheses have not been reported. We concluded that continued investigation of the biological activities of stilbenoids **1**-**3** must therefore rely on material isolated from natural sources, at least in the short term. In order to complete comprehensive structure and pharmacological studies, an

alternative enrichment and efficient large-scale isolation scheme was developed that has broader application to the purification of stilbenoids, in general.

It has been shown above, that acid hydrolysis could produce **1** in high yield from the more abundant glycosylated compound **2**. Compound **1** is slightly more active than **2** and **3** and consequently, we explored enrichment of **1** by enzymatic hydrolysis of **2** prior to isolation. In a procedure inspired by the enzymatic hydrolysis of ruberythric acid to alizarin in madder root,[53] the biotransformation of **2** to **1**was achieved by utilizing the endogenous enzymes present in the leaf. The dried ground leaf material of *A. polyandra* was suspended in tap water for 7 days at 35°C. Following chromatographic purification, a sixteen-fold enrichment of **1** (0.08–1.3% dry wt.), but not complete conversion, was achieved over this time-frame.

The Genus *Anisoptera* comprises 11 species [The Plant List[54]], that are distributed from Bangladesh to New Guinea,[55] and eight of these species are currently listed as endangered, or critically endangered.[56] *Anisoptera* belongs to the subfamily Dipterocarpoideae which are represented by resinous rainforest trees, which characteristically produce resveratrol oligostilbenes.[57] The chemistry of only two *Anisoptera* species has been investigated: bergenin and six resveratrol oligomers has been reported from *A. marginata* [58, 59]; whilst the chemical characterization of *A. scaphula* is ongoing.[60]

Stilbenoids are biologically active phytoalexins, and their range of pharmacological activity has been comprehensively reviewed.[46] Of relevance to this study, is that the *tetrameric* stilbenes have demonstrated antifungal,[43] antibacterial,[61] antiviral,[62] anti-inflammatory,[63] cytotoxic[64] and antitumor activity *in vitro*.[65] The biological activity of hemsleyanol C, an epimer of **3**, is noted as a potent topoisomerase II inhibitor,[66] whilst an epimer of **1**, has demonstrated moderate antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA).[61]

In this study we have identified three resveratrol tetramers from two *Anisoptera* species, which inhibit YopE and YopH from *Y. pseudotuberculosis in vitro*. As these compounds did not inhibit the growth of *Y. pseudotuberculosis* (Table 1), these compounds may selectively inhibit the

T3SS. It has been reported that T3SS inhibitors are able to attenuate virulence without effecting bacterial growth in *Y. pseudotuberculosis*.[20] This is the first report of chemical constituents of *A. thurifera* and *A. polyandra*, and the first report detailing the bioassay-directed isolation of stilbenoid oligomers against protein components of the T3SS. The promising *in vitro* data for stilbenoids **1** warranted further biological evaluation, the anti-infective activity of **1** against three Gram-negative bacterium are reported by co-workers elsewhere.[52]

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded at 30 ^{o}C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer (Varian, Walnut Creek, CA, USA). The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for acetone-d_6 (δ_H 2.05 and δ_C 29.9) or CD_3OD (δ_H 3.31 and δ_C 49.15). LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters, Milford, MA, USA). HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer (Bruker, Karlsruhe, Germany). A BIOLINE orbital shaker (Edwards Instrument Company, Narellan, NSW, Australia) was used for the large-scale extraction of plant material. Machery Nagel Polyamide CC6 (0.05–0.016 mm) was used for tannin/polyphenolic removal (Machery Nagel, Düren, Germany). Alltech Davisil 40–60 μm 60 Å C_{18} bonded silica was used for pre-adsorption work (Alltech, Deerfield, IL, USA). Merck 40–63 μm silica (Kiesselgel 60) was used for flash chromatography. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler (Waters, Milford, MA, USA) were used for HPLC. A ThermoElectron C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) (Thermo Scientific, Los Angeles, CA, USA) and a Phenomenex Luna C_{18} column 5 μm 143 Å column (Phenomenex, Torrance, CA, USA) were used for semi-preparative (21.2 mm × 250 mm) and preparative (50 × 150 mm) HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered (Millipore, Billerica, MA,

USA). All synthetic reagents were purchased from Sigma Aldrich and used without further purification.

Plant Material. The leaves of *Anisoptera thurifera* (Blanco) Blume and *A. thurifera* subsp. *polyandra* (Blume) P.S.Ashton (syn. *Anisoptera polyandra*) were collected in Papua New Guinea. Voucher samples (QID016405; *A. thurifera*) and (QID018040; *A. polyandra*) have been lodged with the Biodiversity Research Pty Ltd, Port Moresby, Papua New Guinea.

Bioassay-Directed Isolation. In separate extraction processes, the air-dried and ground leaves of *A. thurifera* (10 g) and *A. polyandra* (10 g) were defatted (250 mL *n*-hexane, 2 h) and then sequentially extracted with CH_2Cl_2 (250 mL, 2 h) and CH_3OH (2 × 250 mL, 2 h then 24 h) with stirring (200 rpm). The filtered CH_2Cl_2 and CH_3OH extractions were combined and dried under reduced pressure to yield a crude extracts (1.9 g, *A. thurifera*, and 2.0 g *A. polyandra*).

The organic extracts were resuspended in CH_3OH (150 mL) and loaded onto a polyamide gel column (30 g bed volume, pre-equilibrated with CH_3OH) to remove pigments. The column was eluted with CH_3OH (300 mL), and the eluent evaporated to obtain a crude extract (1.0 g, *A. thurifera*, and 1.1 g, *A. polyandra*). A portion of this crude material (0.8 g) was pre-adsorbed to C_{18}-bonded silica (1 g) then packed into a stainless steel guard cartridge (10 × 30 mm) that was subsequently attached to a C_{18} semi-preparative HPLC column. Isocratic HPLC conditions of H_2O-CH_3OH-CF_3COOH (90:10:0.1) were initially employed for the first 10 min, then a linear gradient to CH_3OH (0.1% CF_3COOH) was run over 40 min, followed by isocratic conditions of CH_3OH (0.1% CF_3COOH) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the run then submitted to bioassay.

In the case of *A. thurifera*: Bioassay data indicated that fractions 25–30 were the active fractions. ^{1}H NMR analysis of the fractions identified that fractions 25–26 contained vatalbinoside A (**2**, 44.7 mg, 1.0% dry wt). Fractions 27–30 were a mixture of related oligostilbenes and were combined (73.4 mg) and further fractionated using identical C_{18} HPLC conditions to those detailed above. ^{1}H NMR analyis of all UV-active peaks resulted in the identification of the previously

reported natural products, vatalbinoside A (**2**, 4.2 mg, 0.10% dry wt), vaticanol B (**3**, 10.8 mg, 0.25% dry wt) and (–)-hopeaphenol (**1**, 3.4 mg, 0.08% dry wt).

In the case of *A. polyandra*: Bioassay data indicated that fractions 29–31 were active. Fractions 29–31 were combined (32.7 mg), the mixtures were fractionated using identical C_{18} HPLC conditions to those detailed above. ^{1}H NMR analysis of all UV-active peaks resulted in the identification of the previously reported natural product, the vaticanol B (**3**, 19.2 mg, 0.26% dry wt). Compounds **1** and **2** were identified in extracts of *A. polyandra* but were not isolated in sufficient quantities during the bioassay-directed fractionation step.

Alternative Enrichment of 1 and Optimized Large-scale Purification of 1–3. Several strategies, with broader application, to the optimal enrichment or semi-purification of the major stilbenoid tetramers prior to HPLC purification were developed.

Enzymatic Hydrolysis of Stilbenoid Glycosides using Endogenous Leaf Enzymes. The air-dried and ground leaf material of *A. polyandra* (100 g) was suspended in tap water (400 mL) in a glass container. The leaf material/water was maintained at 35 °C in a water bath for 7 days. The water level was maintained at 400 mL with the addition of fresh tap water. Prior to filtration, methanol (400 mL) was added to the plant material to solubilize the aglycone product. The sample was filtered through a glass frit (Pore 1) then sequentially extracted with acetone (2 × 400 mL) at ambient temperatures for 24 h with shaking. The resulting H_2O/CH_3OH and acetone extracts were filtered then combined and dried under reduced pressure to yield a crude extract (14.5 g).

Enrichment of Stilbene Glycosides and Stilbene Aglycones by Normal Phase Column Chromatography. The aglycone-enriched extract of the leaf material was resuspended in acetone and pre-adsorbed to silica gel (100 g). The dry pre-adsorbed sample was loaded onto a silica flash column (200 g, 12.5 cm diam. × 4 cm high; pre-equilibrated with 1:1 hexane:EtOAc. The extract was fractionated using a step-wise gradient consisting of: 1:1 hexane:EtOAc; 1:9 hexane:EtOAc; 19:1 acetone:CH_3OH. Fraction 1, which contained plant pigments, was discarded. Fraction 2

contained a crude mixture of stilbenoid aglycones and fraction 3 contained a crude mixture of stilbenoid glycosides.

Purification of Compounds 1 – 3 by Reversed Phase Preparative HPLC. Fraction 2 (10.3 g) was re-suspended in CH_3OH and pre-adsorbed onto C_{18} bonded silica (40 g). The dried pre-adsorbed extract (sufficient for three guard cartridges) was then loaded into a stainless steel guard cartridge (50 × 25 mm diam.). A C_{18} Betasil preparative column (Thermo Electron Company Betasil C18; 150 × 50 mm; 5 μm) was pre-equilibrated with the starting mobile phase H_2O-CH_3OH-CF_3COOH (85:15:0.1). Isocratic HPLC conditions of H_2O-CH_3OH-CF_3COOH (85:15:0.1) were employed for the first 2 min, then a linear gradient to H_2O-CH_3OH-CF_3COOH (63:37:0.1) was run over 32 min, followed by isocratic conditions of H_2O-CH_3OH-CF_3COOH (63:37:0.1) for a further 51 min, and lastly, a linear gradient to H_2O-CH_3OH-CF_3COOH (20:80:0.1) over 68 min, all at a flow rate of 18 mL/min. 118 Fractions (84 sec.) were collected over 150 min from the start of the run. Pure (–)-hopeaphenol (**1**) eluted between fractions 26 – 40 (1.02 g, 99% purity; 0.5 g 60% purity; 1.3% dry wt., cumulative yield). Vaticanol B (**3**) eluted between fractions 41–72 (4.15 g, ca. 70–99% purity; ca. 2.9% yield dry wt.).

Fraction 3 contained a moderate amount of the unhydrolyzed β-glycoside **2** (1.7 g, 30% purity; 0.5% yield dry wt.). Crystals of **2** was obtained after further purification by reversed-phase HPLC. A portion of this crude material (0.1 g) was pre-adsorbed to C_{18}-bonded silica (1 g) then packed into a stainless steel cartridge (10 × 30 mm) that was subsequently attached to a C_{18} Betasil semi-preparative HPLC column (21.2 mm × 150 mm). A linear gradient from H_2O-CH_3OH-CF_3COOH (80:20:0.1) to H_2O-CH_3OH-CF_3COOH (72:28:0.1) was run over 30 min, followed by isocratic conditions of H_2O-CH_3OH-CF_3COOH (72:28:0.1) for a further 30 min, all at a flow rate of 9 mL/min. Compound **2** eluted between 36–42 min. Crystalline **2** (not suitable for X-ray crystallographic studies) was obtained by slow evaporation of the mobile phase.

*(–)-Hopeaphenol (**1**):* Colourless plates (CH_3OH /H_2O/0.1% CF_3COOH); decomp. ~280°C; Lit: 351°C (evac. cap.)[49]; $[a]_D^{25}$= -454° (*c* 0.1, CH_3CH_2OH); Lit: $[a]_D^{20}$= -407° (*c* not specified,

CH_3CH_2OH)[49]; ^1H and ^{13}C NMR data were consistent with literature values[48]; (+)-LRESIMS (rel. int.) *m/z* 907 (100) $[M+H]^+$. Crystals of the monohydrate of **1** suitable for X-ray diffraction studies were obtained by slow evaporation of the CH_3OH/H_2O.0.1% CF_3COOH HPLC eluent of **1**.

*Vatalbinoside A (**2**):* Colourless needles decomp. ~280 °C; $[a]_D^{25}$= -341° (*c* 0.1, CH_3OH); Lit: $[a]_D^{25}$= -320° (*c* 0.1, CH_3OH)[44]; ^1H and ^{13}C NMR data were consistent with literature values[44]; IR ν_{max} (KBr) 3265, 1612, 1513, 1345, 1240, 1022, 839 cm^{-1}; (+)-LRESIMS (rel. int.) *m/z* 1091.4 (100) $[M+Na]^+$.

*Vaticanol B (**3**):* white amorphous powder; m.p. decomp. ~280 °C; $[a]_D^{25}$= -46° (*c* 0.06, CH_3OH); Lit: $[a]_D^{20}$= -14 (*c* 0.1, CH_3OH)[51]; ^1H and ^{13}C NMR data were consistent with literature values[51]; (+)-LRESIMS (rel. int.) *m/z* 907 (100) $[M+H]^+$, (+)-HRESIMS *m/z* 907.2740 ($C_{62}H_{52}O_{17}$ $[M+H]^+$ requires 907.2749).

Acid Hydrolysis of 2. A solution of **2** (10 mg in 1 mL of 5% H_2SO_4 in CH_3CH_2OH) was refluxed for 4 h at 65 °C. The reaction mixture was allowed to cool, then evaporated to dryness. The crude reaction mass was partitioned between EtOAc (2 × 10 mL) and sat. $NaHCO_3$ (10 mL). The organic layer was washed with brine solution, and subsequently dried over Na_2SO_4. The organic layer was evaporated and the reaction mixture was further purified by C_{18} preparative HPLC using the conditions described for fraction 3 in §4.4.3. to yield (–)-hopeaphenol (**1**, 8 mg, 93% yield, 98% purity).

Methylation of (–)-Hopeaphenol (1). (–)-Hopeaphenol (**1**, 20 mg, 0.02 mmol) was dissolved in CH_3OH–CH_2Cl_2 (1:1, 1.5 mL) at room temperature before TMS-diazomethane (2.0 M in Et_2O, 650 µL, 1.30 mmol) was added dropwise.[67] The reaction was stirred overnight at room temperature, and then evaporated to dryness. The crude reaction mixture was adsorbed to C_{18}-bonded silica then purified by C_{18} semi-preparative HPLC (Betasil column, 21.2 mm × 150 mm). A linear gradient from H_2O-CH_3OH-CF_3COOH (50:50:0.1) to H_2O-CH_3OH-CF_3COOH (5:95:0.1) was run over 30 min, followed by a linear gradient to CH_3OH (0.1% CF_3COOH) over 10 min

followed by isocratic conditions of CH_3OH (0.1% CF_3COOH) for a further 20 min, all at a flow rate of 9 mL/min.

*(–)-Hopeaphenol permethyl ether (**4**):* (15.6 mg, 68% yield, ≥95% purity) eluted between 33–37 minutes. m.p. 169-172 °C (CH_3OH-H_2O); Lit: 162–164°C (benzene-CH_3OH)[49]; $[a]_D^{25}$= -387° (*c* 0.1, CH_3OH); Lit.: $[a]_D^{RT}$ -378° (*c* not specified, $CHCl_3$)[49]; ^{1}H NMR (500 MHz, CD_3OD): *δ* 3.29 (s, H-13a-OMe/13d-OMe), 3.65 (s, H-13b-OMe/13c-OMe), 3.72 (s, H-4b-OMe/4c-OMe), 3.77 (s, H-11a-OMe/11d-OMe), 3.80 (s, H-4a-OMe/4d-OMe), 3.87 (brs, H-8b/8c), 4.15 (d, 11.8, H-8a/8d), 4.92 (d, 2.4, H-14b/14c), 5.73 (brs, H-7b/7c), 5.77 (d, 11.8, H-7a/7d), 5.84 (d, 2.4, H-12b/12c), 6.41 (d, 2.4, H-14a/14d), 6.66 (d, 2.4, H-12a/12d), 6.71 (d, 8.6, H-3b,5b/3c,5c), 6.85 (d, 8.7, H-3a,5a/3d,5d), 6.89 (d, 8.4, H-2b,6b/2c,6c), 7.14 (d, 8.7, H-2a,6a/2d,6d); ^{13}C NMR (126 MHz, CD_3OD); 41.5 (C-7b/7c),48.3 (C-8b/8c), 50.8 (C-8a/8d), 55.9 (C-11a-OMe/11d-OMe), 55.9 (C-4b-OMe/4c-OMe), 56.0 (C-4a-OMe/4d-OMe), 56.1 (C-13a-OMe/13d-OMe), 56.6 (C-13b-OMe/13c-OMe), 89.0 (C-7a/7d), 95.8 (C-12b/12c), 96.9 (C-12a/12d), 105.0 (C-14a/14d),108.8 (C-14b/14c), 114.5 (C-3b,5b/3c,5c), 115.1 (C-3a,5a/3d,5d), 120.7 (C-10b/10c), 124.3 (C-10a/10d), 129.5 (C-2b,6b/2c,6c), 130.3 (C-2a,6a/2d,6d), 132.2 (C-1a/1d), 136.2 (C-1b/1c), 140.3 (C-9b/9c), 142.6 (C-9a/9d), 159.2 (C-4b/4c), 159.8 (C-11b/11c), 160.9 (C-13a/13d), 161.2 (C-4a/4d), 161.7 (C-11a/11d), 161.8 (C-13b/13c); (+)-LRESIMS (rel. int.) *m/z* 1048 (100) $[M+H]^+$; HRESIMS *m/z* 1047.4307 ($C_{66}H_{63}O_{12}$ $[M+H]^+$ requires 1047.4319).

Methylation of Vaticanol B (3). Vaticanol B (**3**, 22.1 mg, 0.024 mmol) and K_2CO_3 (320 mg, 2.32 mmol) were dissolved in dry acetone (5 mL) at room temperature before MeI (200 μL, 3.2 mmol) was added dropwise. The reaction was refluxed for 2 h at 40°C then evaporated to dryness. The crude reaction mixture was adsorbed to C_{18} bonded silica then purified using the same conditions described above for compound **4**. Evaporation of fractions 35–38 yielded the crystalline vaticanol B permethyl ether (**5**) suitable for X-ray diffraction studies.

*Vaticanol permethyl ether (**5**):* colourless prisms (21.0 mg, 90% yield, ⩾95% purity); m.p. 240–242°C (CH_3OH-H_2O); $[a]_D^{25}$= +60° (*c* 0.01, CH_3CH_2OH) tentative value only, this compound

has solubility issues in a range of solvents; ^{1}H and ^{13}C NMR data were consistent with literature values [51]; (+)-LRESIMS (rel. int.) *m/z* 1048 (100) $[M+H]^{+\cdot}$

X-ray Crystallography. Unique data sets for compounds **1** (Cu-K_α radiation) as the monohydrate and **5** (Mo-K_α radiation) were measured at 200 K on an Oxford-Diffraction GEMINI S Ultra CCD diffractometer utilizing CrysAlis software.[68] The structures were solved by direct methods and refined by full matrix least squares refinement on F^2. Anisotropic thermal parameters were refined for non-hydrogen atoms; $(x, y, z, U_{iso})_H$ were included and constrained at estimated values. Conventional residuals at convergence are quoted; statistical weights were employed. Computation used, SIR-97[69], SHELX97[70], ORTEP-3[71] and PLATON[72] programs and software systems. The absolute configuration of **1** was determined by anomalous dispersion effects [3975 Bijvoet pairs, Flack parameter -0.05(12)].[73] In the absence of significant anomalous scatterers in compound **5**, Friedel equivalents were merged with the absolute configuration predicted on the basis of optical activity.

*Crystal data for (–)-hopeaphenol (**1**)*: $C_{56}H_{42}O_{12}.H_2O$, M_r = 924.9. Orthorhombic, space group $P2_12_12_1$, a = 11.1051(2), b = 18.7895(4), c = 22.3006(4) Å, V = 4653.2(2)(2) $Å^3$. D_c (Z = 4) = 1.32 g cm^{-3}. μ_{Cu} = 0.77 mm; specimen: 0.36 × 0.23 × 0.08 mm; $'T'_{min/max}$ = 0.768/0.941. $2\theta_{max}$ = 71.2°; N_t = 32588, N = 8969 (R_{int} = 0.045), N_o = 7876; $R1$ = 0.037, $wR2$ = 0.095; S = 1.03, x_{abs} = -0.05(12).

*Crystal data for vaticanol B permethyl ether (**5**):* $C_{66}H_{62}O_{12}$, M_r = 1047.2. Monoclinic, space group $P2_1$, a = 15.5983(6), b = 12.4215(3), c = 16.8136(7) Å, β = 117.230(5), V = 2896.7(2) $Å^3$. D_c (Z = 2) = 1.20 g cm^{-3}. μ_{Mo} = 0.08 mm; specimen: 0.54 × 0.35 × 0.10 mm; $'T'_{min/max}$ = 0.99/0.96. $2\theta_{max}$ = 55.0°; N_t = 22650, N = 6728 (R_{int} = 0.044), N_o = 3205; $R1$ = 0.057, $wR2$ = 0.117; S = 0.84.

Full .cif depositions reside with the Cambridge Crystallographic Data Centre, CCDC Nos. 998909 [(–)-hopeaphenol, **1**] and 953125 (vaticanol B permethyl ether, **5**). Copies can be obtained free of charge on application at the following address: http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi).

Determination of YopE Expression using a Reporter-gene Assay. YPIII(pIB102-Elux)[74] from a culture grown on LB agar containing 25 μg/mL chloramphenicol (Sigma) for 48 h at 26 °C was used to inoculate a liquid culture, then grown for 12–16 h in Brain Heart Infusion Broth (BHI; Difco) containing 25 μg/mL chloramphenicol (Sigma) on an orbital shaker at 26 °C. Liquid cultures were diluted to an OD_{620} of 0.2, then further diluted 1 in 4 in Ca^{2+} depleted media (BHI media with 5 mM EGTA and 20 mM $MgCl_2$) before addition of 30 μL of bacteria to a 384-well white solid lidded Optiplate™ (Perkin Elmer). Before bacterial addition, 5 μL of fraction or compound/controls were added to the plate, by diluting plates of library fractions or compounds/controls in DMSO with a Minitrak™ (PerkinElmer) liquid handler, by addition of 1 μL of fraction to 4 μL of H_2O.

Plates were incubated at 26 °C for 1 h followed by incubation at 37 °C for 3 h and then transferred back to 26 °C for 15 min. 15 μL of 0.1% decanal (Sigma) emulsified in H_2O was added to each well. Plates were read on a Trilux (PerkinElmer) counter using a luminescence protocol. A dose response of streptomycin was used as an antibacterial control. Bacteria in BHI with 2.5 mM Ca^{2+} was used as an in plate negative control for the assay since no Yop protein is expressed during these conditions. Ca^{2+} depletion at 37 °C stimulates Yop production and the Yop production will eventually suppresses growth.[15]

Fractions were tested at single point concentrations of 7.14 μge/μL Active fractions and controls were retested at 7.14, 1.42, 0.71, 0.14 and 0.071 μge/μL. Pure compounds were screened at 10, 20, 50 and 100 μM. YopE suppression was calculated using linear regression on the basis of the reduction in luminescent signal versus sample concentration (μg/mL). IC_{50} values were calculated from interpolation of the dose response curves.

Combined Reporter-gene and Phosphatase Assay for the Determination of YopH Secretion. Before addition of decanal, and as according to the YopH protocol, 5 μL of the final assay volume was added to a clear 384-well plate (Becton Dickinson) containing 45 μL of YopH substrate *p*-nitrophenyl phosphate (pNPP; Acros Organics; 12.5 mM in 20 mM MES pH 5.0 and 0.8

mM DTT), with a Biomek FX liquid handler (Beckman Coulter). Plates were incubated for 15 min at 37 °C before addition of 10 µL of sodium hydroxide to stop the reaction. Plates were then read at 405 nm on a VictorII Wallac plate reader (PerkinElmer). To the remainder of the assay volume decanal was added and luminescence was measured as described above. Controls were as described for the reporter-gene assay. Fractions were tested at single point concentrations of 7.14 μge/μL Active fractions and controls were retested at 7.14, 1.42, 0.71, 0.14 and 0.071 μge/μL. Pure compounds were screened at 10, 20, 50 and 100 μM. IC_{50} values were calculated from interpolation of the dose response curves.

Antibacterial Optical Density (OD_{620}) Assay. YPIII(pIB102-Elux) cultures were grown overnight, then diluted to an OD_{620} of 0.2 in BHI medium, with 2.5 mM Ca^{2+}. A further 1:4 dilution in BHI with 2.5 mM Ca^{2+} was prepared before addition to the assay. 50 μL of the diluted bacteria was added to clear, lidded 384 well plates (Becton Dickinson), containing 5 μL of fraction/compound or controls. Active fractions and controls were screened at a single point concentration of 7.14 μge/μL then retested at 4.45, 0.91, 0.45, 0.091 and 0.045 μge/μL to determine a dose response. Plates were incubated for 3 h at 37 °C and then transferred to room temperature for 15 min before reading at 620 nm on a VictorII Wallac plate reader. Streptomycin was used as an in-plate negative growth control and an external plate contained a dose response of streptomycin for the estimation of the antibacterial IC_{50} values. Pure compounds were screened at 10, 20, 50 and 100 μM.

ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra for (–)-hopeaphenol permethyl ether (**4**) and an ORTEP view of the dibromodeca-*O*-methyl derivative of hopephenol. This material is available free of charge via the Internet at http://pubs.acs.org.

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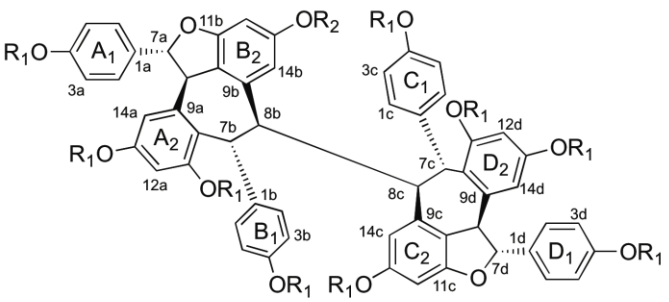
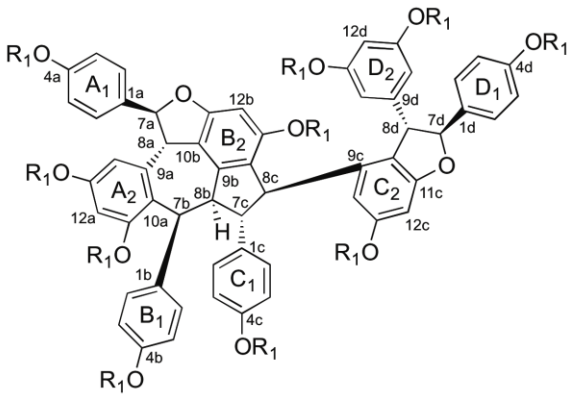


R_1 = H R_2 = H (-)-Hopeaphenol (**1**)
R_1 = H R_2 = β-D-glucose Vatalbinoside A (**2**)
R_1 = Me R_2 = Me (-)-Hopeaphenol permethyl ether (**4**)



R_1 = H Vaticanol B (**3**)
R_1 = Me Vaticanol B permethyl ether (**5**)

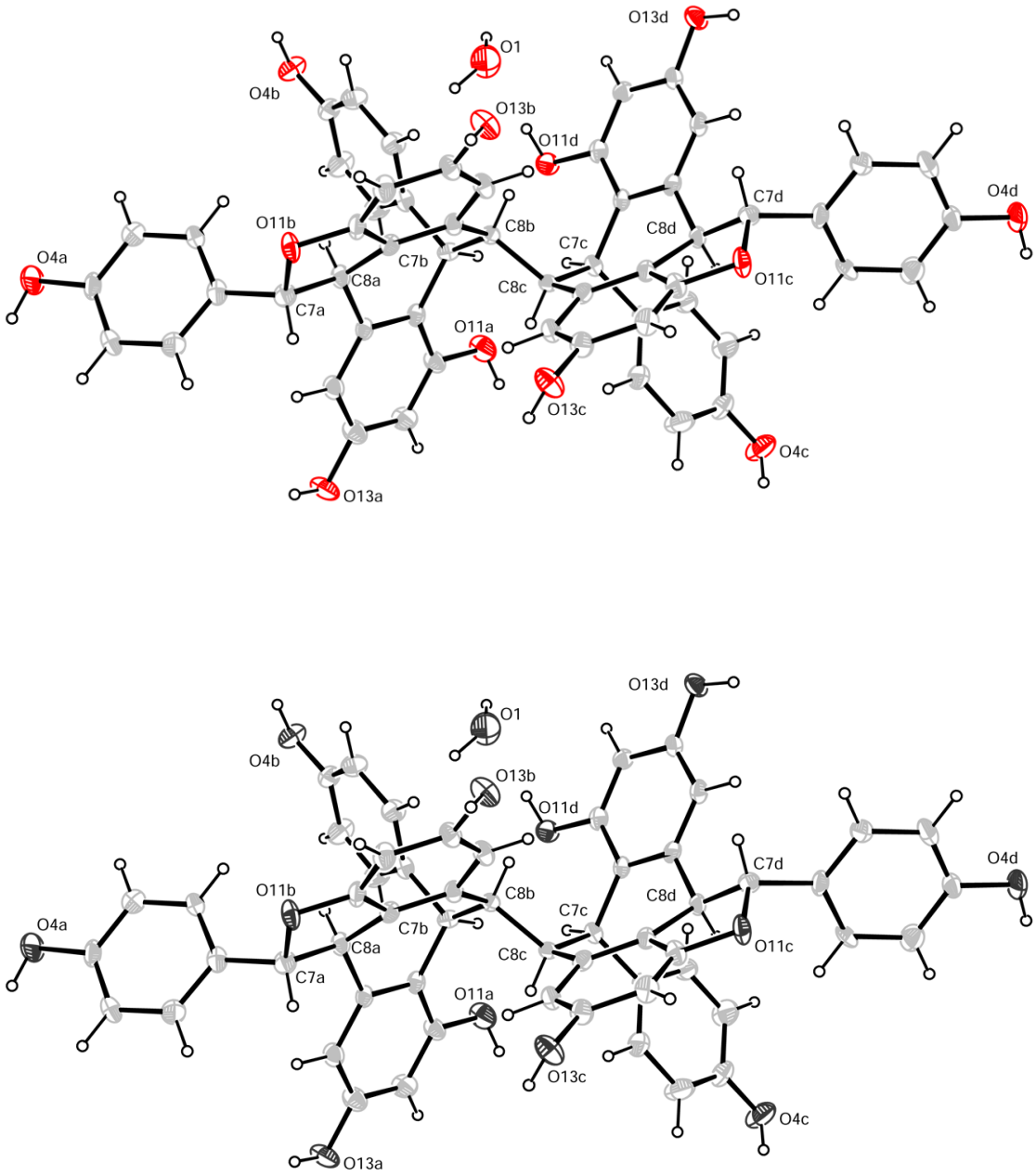


Figure 1. ORTEP view of the compact tetrameric resveratrol rotamer (–)-hopeaphenol (**1**).
The C1 – C7 – C8 – C9 torsion angles for each ring system in **1** are: A -85.6(2)°; B 66.1(2)°; C 64.2 (2)°; and D -88.8(2)° compared to the values of: A -83, -88°; B 60, 60°; C -60,60°; and D -83, -88° structure of the dibromodeca-*O*-methyl derivative (in the supporting information S7).[50]

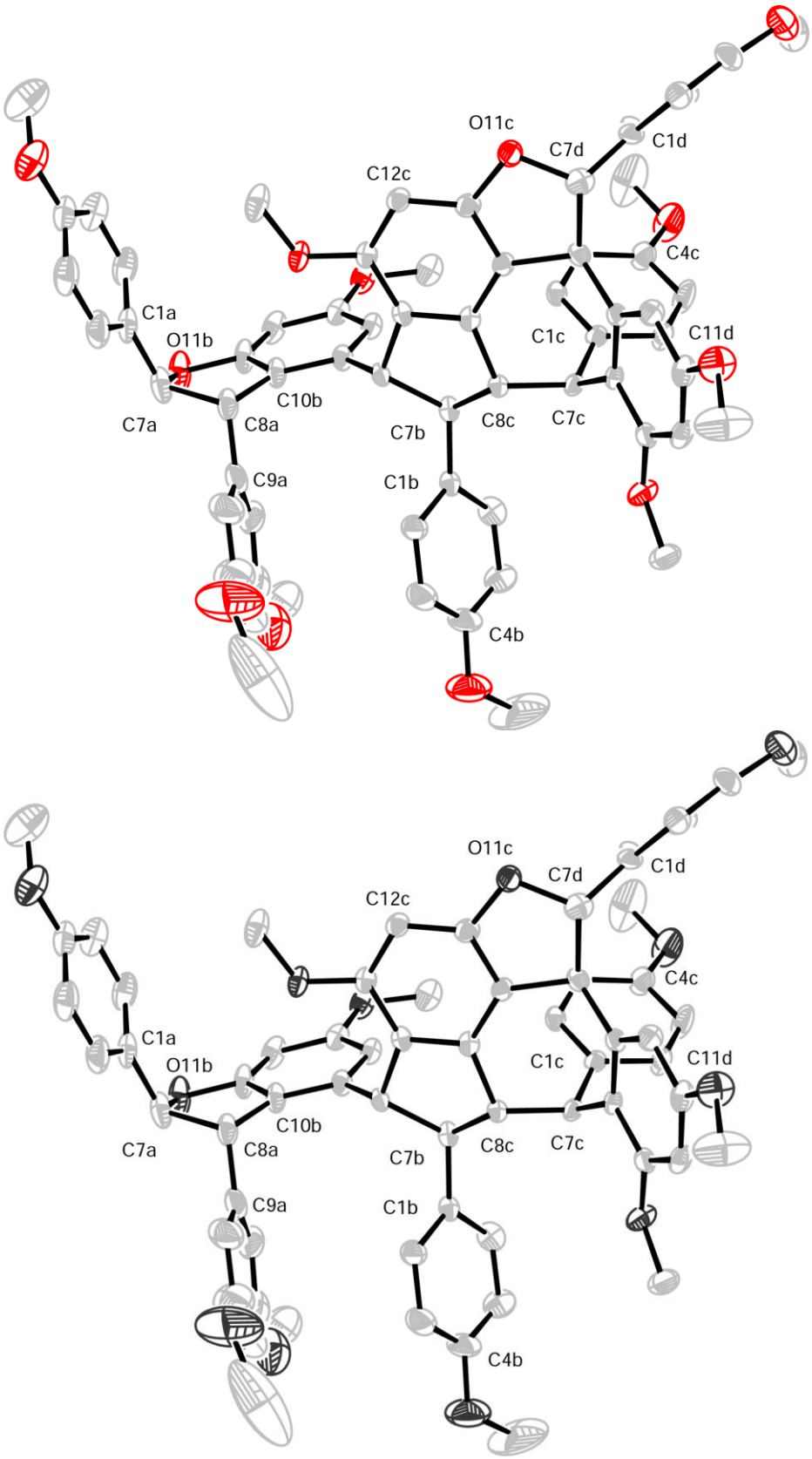


Figure 2. ORTEP view of the extended tetrameric resveratrol rotamer vaticanol B permethyl ether (**5**) The C1–C7–C8–C9 torsion angles for each ring system in **5** are: A -98.9(5)°; B -59.8(6)°; C 156.8(4)°; and D -138.9(5)°.

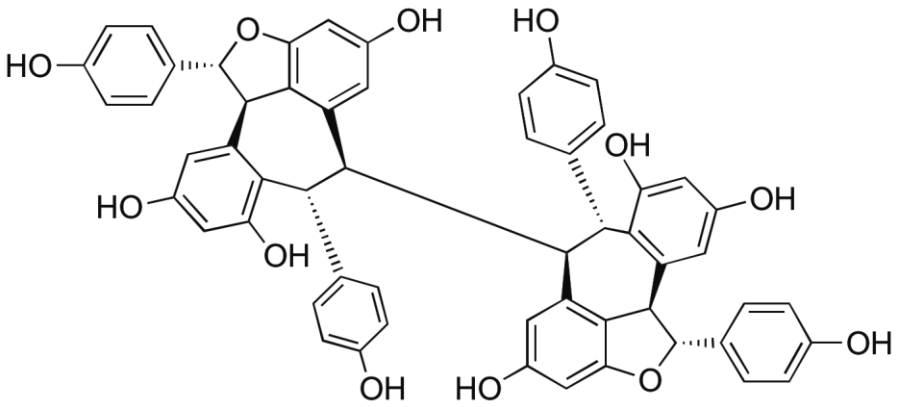
.

Table 1. The YopE, YopH anti-infective and antibacterial activities of compounds **1**–**3**

Compound	IC_{50} (μM)		
	YopE	YopH	Antibacterial assay [a]
1	8.8	2.9	Not active [b]
2	12.5	4.5	Not active [b]
3	9.9	3.3	Not active [b]
Streptomycin[c]	38.0	31.0	36.0

[a] *Y. pseudotuberculosis* optical density assay; [b] at 364 μM; [c] commercially available antibiotic used as positive control.

TOC Graphic



(-)-Hopeaphenol

IC_{50} = 8.8 μM (YopE)
IC_{50} = 2.9 μM (YopH)