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Development of mitochondria-targeted derivatives of resveratrol

Lucia Biasutto [a,b,†], Andrea Mattarei [a,†], Ester Marotta [a], Alice Bradaschia [b], Nicola Sassi [b], Spiridione Garbisa [b], Mario Zoratti [b,c,*], Cristina Paradisi [a]

[a] Department of Chemical Sciences, Università di Padova, via Marzolo 1, 35131 Padova, Italy
[b] Department of Biomedical Sciences, Università di Padova, viale G. Colombo 3, 35131 Padova, Italy
[c] CNR Institute of Neuroscience, viale G. Colombo 3, 35131 Padova, Italy

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ABSTRACT

To target natural polyphenols to the subcellular site where their redox properties might be exploited at best, that is, mitochondria, we have synthesised new proof-of-principle derivatives by linking resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) to the membrane-permeable lipophilic triphenylphosphonium cation. The new compounds, (4-triphenylphosphoniumbutyl)-4′-*O*-resveratrol iodide and its bis-acetylated derivative, the latter intended to provide transient protection against metabolic conjugation, accumulate into energized mitochondria as expected and are cytotoxic for fast-growing but not for slower-growing cells. They provide a powerful potential tool to intervene on mitochondrial and cellular redox processes of pathophysiological relevance.

Plant polyphenols are the object of intense interest because they display, at least in vitro, properties and effects of relevance for physiopathological conditions ranging from aging to cancer. These effects are ascribed to their redox properties and to interactions with signaling proteins. Polyphenols can act either as anti- or pro-oxidants, that is, inhibitors or enhancers of oxidative and radical chain processes.[1] Whether an anti- or a pro-oxidant effect predominates depends, besides the redox potential of the polyphenol, on the abundance of metal ions sustaining a redox cycle ($Fe^{2+/3+}$, $Cu^{+/2+}$) and/or of oxidizing enzymes, on the ion-chelating properties of the molecule, on pH, on the concentration of the polyphenol, and on the subcellular compartment. Either pro-oxidant or anti-oxidant activity may lead to useful oncological applications. Reactive Oxygen Species (ROS) are thought to be a major factor in cancerogenesis.[2] In particular, ROS production by mitochondria[3,4] is emerging as a key factor. The metastatic potential of cell lines has been convincingly related to this parameter.[5] Mitochondrial ROS are involved in the activation of Hypoxia Inducible Factor (HIF),[6,7] which influences angiogenesis and other aspects of tumor development.[7,8] Thus, an anti-oxidant action may limit metastasis and tumor growth. Indeed resveratrol, the representative polyphenol selected for this work, inhibits cell shedding from primary tumors.[9] On the other hand, ROS play fundamental roles in apoptosis[10] and can induce the Mitochondrial Permeability Transition (MPT),[11] promoting in both cases cell death. Cancer cells are constitutively under oxidative stress[4] and an intensification of this stress may lead to their selective elimination. Resveratrol, in addition to other important activities,[12] reportedly exerts anti-proliferative and pro-apoptotic effects on various tumor-derived cells[13,14] and antagonizes growth of xenografts and mutagen-induced cancers.[15] It has been recently shown that resveratrol-induced death of cultured colorectal carcinoma cells involves generation of superoxide anion, that is, pro-oxidant action, at mitochondria.[14] The IC_{50} for death induction was found to be in the hundreds of μM range, a concentration which cannot be reached in vivo due to the poor bioavailability of polyphenols.[16]

We are interested in exploiting the potential of polyphenols through chemical modifications designed to serve specific purposes. Thus, an increase in solubility was achieved via esterification with aminoacids.[17] The present work aims at targeting polyphenols to the subcellular compartment where they are expected to best realize their anti-cancer potential (as well as other functions), that is, mitochondria. We report here the synthesis and properties of new mitochondriotropic derivatives of resveratrol obtained by coupling it to the membrane-permeable lipophilic cation triphenylphosphonium (TPP^+)[18] which drives accumulation in compartments held at negative relative voltage, such as the mitochondrial matrix, according to Nernst's law. Since the mitochondria of cancer cells maintain a higher-then-normal transmembrane potential,[19] mitochondria-targeted drugs may be cancer-selective.

The target derivative **4** was synthesised starting from resveratrol (**1**) in three steps as outlined in Scheme 1.[20] Briefly, O-alkyl-

* Corresponding author. Tel.: +39 049 8276054; fax: +39 049 8276049.
E-mail address: zoratti@bio.unipd.it (M. Zoratti).
† The first two authors contributed equally to the work.

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ation introduces a chlorobutyl group which is then converted to the desired TPP^+ derivative via two consecutive nucleophilic substitution steps: –Cl → –I → – TPP^+I^-. Direct substitution of chloride by triphenylphosphine was unsatisfactory because it required high temperatures which led to some decomposition. The assignment of the site of O-alkylation in **2** is based on ^1H NMR data: a unique signal is found for H-2 and H-6 indicating that these protons are equivalent. The acetylated derivative **6** was also prepared (Scheme 1), so as to compare it with **4** and assess the importance of the free hydroxyl groups for the behavior of these new mitochondriotropic molecules.

The solubility in water of the resveratrol derivatives is $(3.12 \pm 0.20) \times 10^{-5}$ mol L^{-1} and $(9.7 \pm 0.6) \times 10^{-5}$ mol L^{-1} for **4** and **6**, respectively. These solubilities are significantly higher (15- and 45-fold, respectively) than that of resveratrol. Both new compounds are essentially stable in aqueous media: **4** for at least one week both in deionised water and in Hank's Balanced Saline Solution (HBSS) with 10% CH_3CN (added to insure solubility of hypothetical reaction products); **6** for at least 24 h in deionised water, while in HBSS acetyl groups were slowly hydrolised (about 7% conversion to the monoacetylated derivative in 6 h). There were no detectable metabolic modifications of either **4** or **6** by cultured Human Colon Tumor (HCT) 116 cells[20] over 6 h (Fig. 1) or whole freshly drawn rat blood over 75 min (not shown), except for the hydrolysis of the acetyl ester groups of **6** in both cases.

Two methods were used to verify accumulation of the new compounds into mitochondria.[21] First, their uptake by isolated, respiring Rat Liver Mitochondria (RLM) was monitored using a TPP^+-sensitive electrode. A representative experiment with **6** is shown in Figure 2. The introduction of mitochondria causes a decrease (upward deflection of the signal) of **6** in the medium, due to uptake into the mitochondrial matrix. After addition of excess Ca^{2+}, which induces the MPT, or of uncouplers (not shown), **6** is partially released. The release is incomplete presumably due to binding of the resveratrol derivative to mitochondrial constituents. Analogous results were obtained with **4** (not shown).

In the second approach we exploited the spectral properties of **6** and **4**, similar to those of resveratrol itself (Supporting Data, Fig. S1 and S2), to follow their accumulation in the mitochondria of cultured cells by monitoring of their fluorescence upon excitation at 340 nm. Images from one such experiment are shown in Figure 3. After addition of **6** to the medium, intracellular structures become progressively fluorescent due to accumulation of the resveratrol derivative (panel B). Addition of a transmembrane potential ($\Delta\psi_m$)-dissipating protonophore (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)) causes a loss of fluorescence due to efflux of the polyphenol (panel C). Some **6** remains in the cytoplasm of the cells due to the plasma membrane potential maintained by K^+ diffusion. Compound **4** behaved analogously (not shown).

As a first test of potential anti-cancer activity, we verified the effects of **4** and **6**, and of control compounds, on cultured cells (Fig. 4). Controls consisted of the parent polyphenol resveratrol, of the phosphonium salt TriPhenylMethylPhosphonium Iodide (TPMP) and of resveratrol plus this latter compound. We used

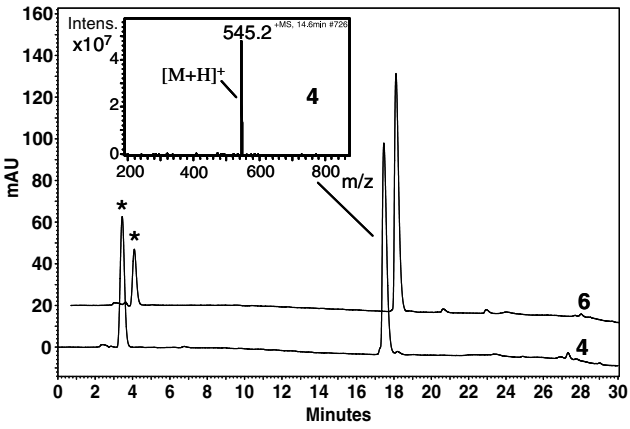
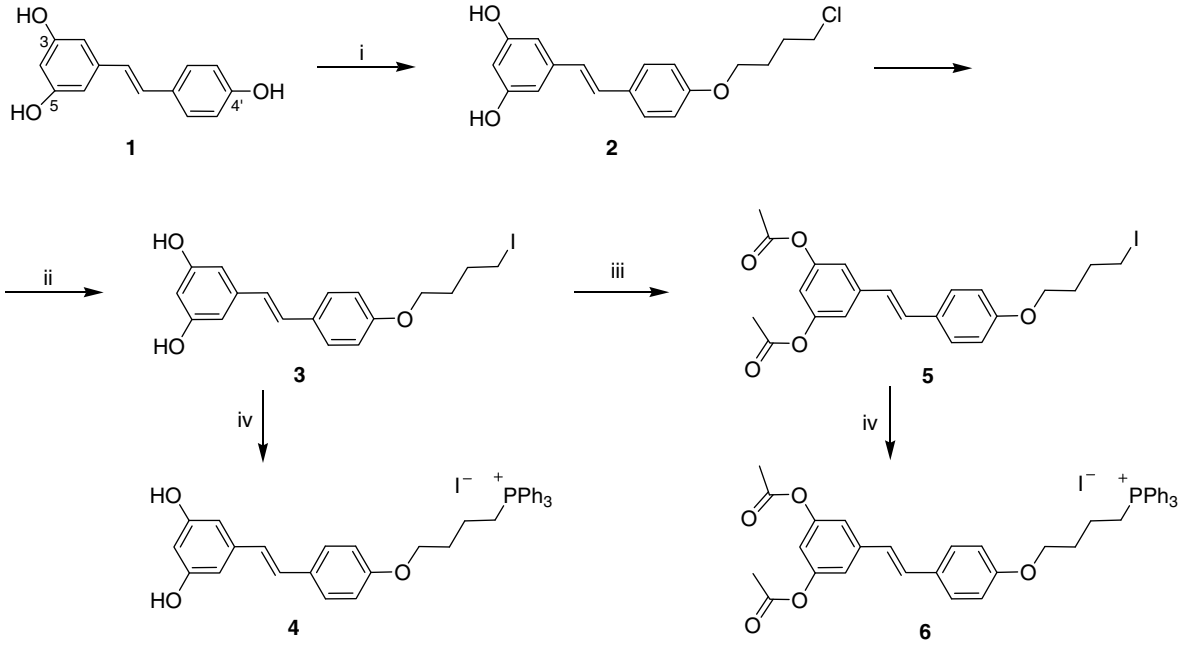


Figure 1. HPLC chromatograms recorded at 320 nm of the extracts obtained after incubation of **4** (lower trace) and **6** (upper trace) with HCT116 cells for 6 h. Inset: positive ESI-MS spectrum of **4**. For clarity, the upper trace was shifted slightly to the right along the time axis: the retention time and the mass spectrum of the major peak in this chromatogram match perfectly those of **4**. Peaks marked with ∗ are due to residual traces of acetone from the sample work-up.



Scheme 1. Synthesis of mitochondriotropic derivatives **4** and **6**. Reagents and conditions: (i) 1-Bromo-4-chlorobutane (1.5 equiv), K_2CO_3 (1.1 equiv), DMF, Ar, rt, 20 h, yield 33%; (ii) NaI, acetone, reflux, 20 h, yield 89%; (iii) $CH_3C(=O)Cl$ (20 equiv), pyr, CH_2Cl_2, yield 73%; (iv) PPh_3 (5 equiv), toluene, 100 °C, 6 h, yield 78%.

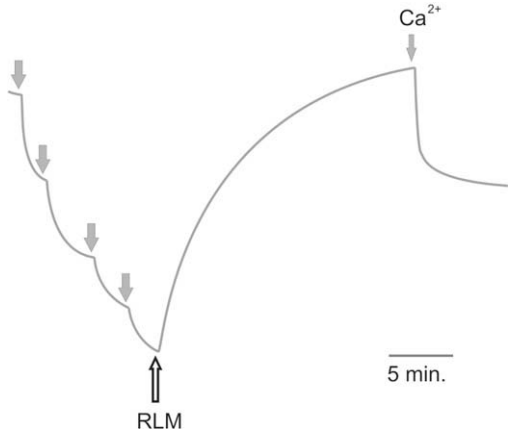


Figure 2. TPP^+-selective electrode response to additions of **6** (thick gray arrows; 0.5 μM each), Rat Liver Mitochondria (RLM) (1 mg prot. mL^{-1}) and $CaCl_2$ (50 μM) at 20 °C.

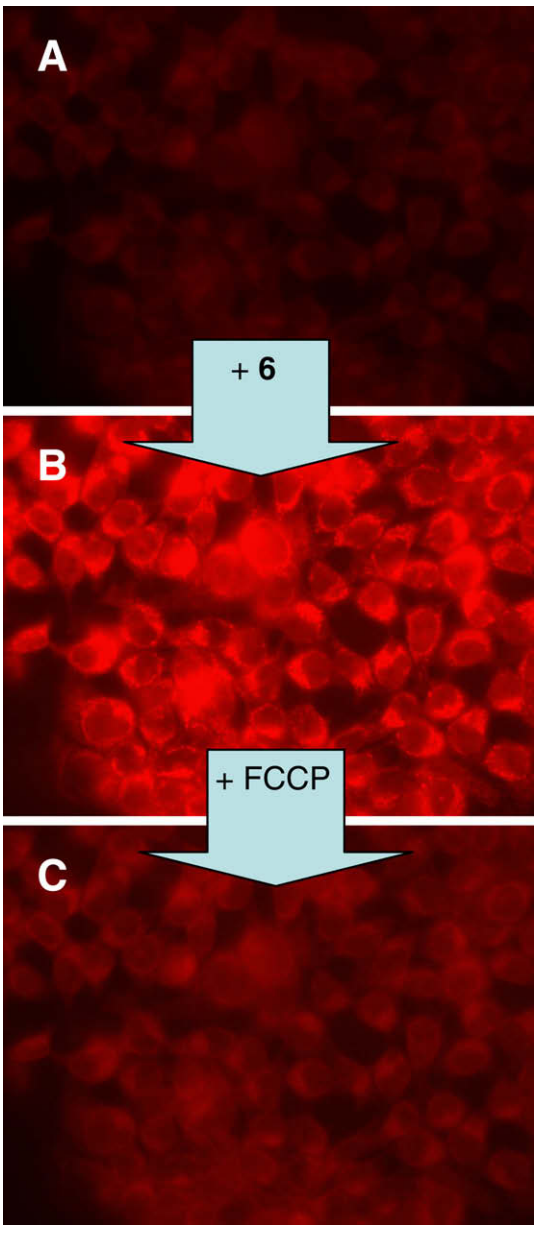


Figure 3. Fluorescence microscope images of cultured HCT116 cells: (A) just before the addition of 10 μM **6**, (B) 25 min after the addition of **6** and just before the addition of 2 μM FCCP, and (C) 10 min after the addition of FCCP.

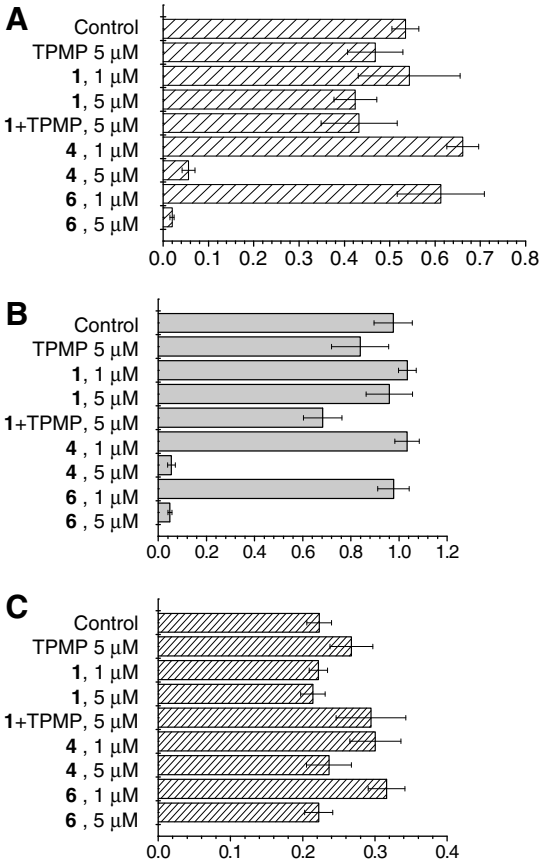


Figure 4. Effect of the mitochondriotropic resveratrol derivatives and control compounds on cell proliferation. Cells were allowed to grow for 3 days in the presence of the specified compounds and assayed using the tetrazolium salt reduction assay (see Supplementary data for details). All measurements were performed in quadruplicate. Averages ± s.d. are given. (A) C-26 mouse colon tumor cells. (B) Fast-growing Mouse Embryonic Fibroblasts (MEF). (C) Slow-growing MEF (note different scale).

the murine colon cancer cell line C-26 and, as controls, fast- and slow-growing non-tumoral mouse embryonic fibroblast (MEF) lines. Cell growth and viability was quantified using the tetrazolium salt reduction (MTT) assay.[21]

The various compounds had little effect on cell proliferation at the 1 μM level. At 5 μM, **4** and **6** displayed a marked cytotoxic effect on the two rapid-growth cell types, that is, C-26 (Fig. 4A) and non-tumoral 'fast' MEFs (Fig. 4B), but not on the slow-growth MEFs (Fig. 4C). Resveratrol, TPMP, and their combination had little effect also at 5 μM and with all three cell types, showing that the activity of the resveratrol-TPP conjugates is not just the sum of the activity of the two components. Selective cytotoxicity for fast-growing cells is characteristic of many chemotherapeutic drugs.

In conclusion we have produced mitochondriotropic resveratrol derivatives with good solubility and stability in aqueous media, which accumulate as expected in regions at negative potential. A class of natural compounds with useful properties can now be targeted to subcellular compartments where they ought to realize their biomedical potential in full. The results of initial cytotoxicity assessments encourage further experimentation in vivo to determine absorption, pharmacokinetics and possible anti-tumoral action.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.100.

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20. Synthetic procedures and characterization.
4′-(4-O-chlorobutyl) resveratrol (**2**). K_2CO_3 (1.33 g, 9.64 mmol, 1.1 equiv) and 1-bromo-4-chlorobutane (2.25 g, 13.14 mmol, 1.5 equiv) were added under argon to a solution of resveratrol (**1**) (2.00 g, 8.76 mmol) in DMF (10 mL). After stirring overnight, the mixture was diluted in EtOAc (100 mL) and washed with 1 N HCl (3 × 50 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2/EtOAc 85:15 as eluent to afford 0.930 g of **2** (33%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.76–1.98 (m, 4H, CH_2), 3.72 (t, 2H, CH_2), 4.02 (t, 2H, CH_2), 6.12 (t, 1H, H-4, J = 2.0 Hz), 6.40 (d, 2H, J = 2.0 Hz, H-2, H-6), 6.82–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 9.21 (s, 2H, 3-OH, 5-OH); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 26.14 (CH_2), 28.90 (CH_2), 45.19 (CH_2Cl), 66.72 (OCH_2), 104.38, 114.62, 126.62, 127.45, 127.76, 129.62, 139.07, 158.16, 158.49; ESI-MS (ion trap): m/z 319, $[M+H]^+$; HRMS (ESI-TOF): m/z 319.1092; Calcd for $C_{18}H_{19}ClO_3{\cdot}H^+$ 319.1095. Anal.: Calcd for $C_{18}H_{19}ClO_3$ C 67.81, H 6.01; found: C 67.78, H 6.02.
4′-(4-O-iodobutyl) resveratrol (**3**). Compound **2** (500 mg, 1.57 mmol, 1 equiv) was added to a saturated solution of NaI in dry acetone (10 mL) and heated at reflux for 20 h. After cooling, the resulting mixture was diluted in EtOAc (100 mL), filtered and washed with water (3× 30 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2/EtOAc 9:1 as eluent to afford 0.570 g of **3** (89%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.71–2.00 (m, 4H, CH_2), 3.35 (t, 2H, CH_2), 4.01 (t, 2H, CH_2), 6.12 (t, 1H, H-4, J = 2.0 Hz), 6.40 (d, 2H, H-2, H-6, J = 2.0 Hz), 6.82–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 9.21 (s, 2H, 3-OH, 5-OH); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 8.54 (CH_2I), 29.66 (CH_2), 29.76 (CH_2), 66.39 (OCH_2), 104.38, 114.62, 126.61, 127.45, 127.76, 129.62, 139.07, 158.16, 158.49; ESI-MS (ion trap): m/z 411, $[M+H]^+$. Anal.: Calcd for $C_{18}H_{19}IO_3$ C 52.70, H 4.66; found: C 52.75, H 4.66.
4′-(4-O-triphenylphosphoniumbutyl) resveratrol iodide **(4).** A mixture of **3** (500 mg, 1.22 mmol) and triphenylphosphine (1.60 g, 6.09 mmol, 5 equiv) in toluene (15 mL) was heated at 100 °C under argon. After 6 h, the solvent was eliminated at reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL). The solvents were decanted and the procedure repeated 4 more times. The precipitate was then filtered to afford 600 mg of **7** (73%). ^{1}H-NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73 (quintet, 2H, CH_2), 1.92 (quintet, 2H, CH_2), 3.67 (t, 2H, CH_2), 4.06 (t, 2H, CH_2), 6.13 (t, 1H, H-4, J = 1.9 Hz), 6.40 (d, 2H, H-2, H-6, J = 1.75 Hz), 6.83–7.04 (m, 4H, =CH, H-2′, H-6′), 7.50 (d, 2H, H-3′, H-5′, J = 8.75 Hz), 7.71–7.95 (m, 15H, aromatic-H), 9.22 (s, 2H, 3-OH, 5-OH). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 18.48 (CH_2), 20.07 (CH_2), 29.15 (CH_2), 65.93 (OCH_2), 104.38, 114.66, 118.47 (Ph, J(^{13}C/^{31}P) = 85.6 Hz), 126.67, 127.41, 127.73, 129.72, 130.25 (Ph, J(^{13}C/^{31}P) = 12.4 Hz), 133.59 (Ph, J(^{13}C/^{31}P) = 10.1 Hz), 134.93 (Ph, J(^{13}C/^{31}P) = 2.9 Hz), 139.03, 158.00, 158.49; ESI-MS (ion trap): m/z 545, M^+; HRMS (ESI-TOF): m/z 545.2236; Calcd for $C_{36}H_{34}O_3P^+$ 545.2240.
3,5-diacetyl-4′-(4-O-iodobutyl) resveratrol **(5).** A solution of acetyl chloride (1.1 mL, 15 mmol, 20 equiv) in CH_2Cl_2 (20 mL) was added dropwise and under continuous stirring to a mixture of **3** (300 mg, 0.73 mmol, 1 equiv) and anhydrous pyridine (0.85 mL, 10.5 mmol, 15 equiv) in CH_2Cl_2 (20 mL) cooled in dry ice/acetone. The reaction mixture was then allowed to slowly warm up to room temperature. CH_2Cl_2 (50 mL) was added and the organic layer was washed with 1 N HCl (3× 50 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography using CH_2Cl_2:n-hexane 4:1 as eluent to afford 280 mg of **5** (78%). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73–2.02 (m, 4H, CH_2), 2.29 (s, 6H, OAc), 3.38 (t, 2H, CH_2), 4.02 (t, 2H, CH_2), 6.86 (t, 1H, H-4, J = 1.9 Hz), 6.96 (d, 2H, H-2′, H-6′, J = 8.5 Hz), 7.08 (d, 1H, =CH, J = 16.5 Hz), 7.21–7.33 (m, 3H, H-2, H-6, =CH), 7.53 (d, 2H, H-3′, H-5′, J = 8.75 Hz); ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 8.49 (CH_2I), 20.83 (CH_3), 29.62 (CH_2), 29.75 (CH_2), 66.43 (OCH_2), 114.74, 116.79, 124.25, 128.08, 129.10, 130.09, 139.81, 151.15, 158.61, 168.99; ESI-MS (ion trap): m/z 495, $[M+H]^+$; HRMS (ESI-TOF): m/z 495.0664; Calcd for $C_{22}H_{23}O_5I{\cdot}H^+$ 495.0663.
3,5-diacetyl-4′-(4-O-triphenylphosphoniumbutyl) resveratrol iodide **(6)**. A mixture of **5** (200 mg, 0.40 mmol) and triphenylphosphine (525 mg, 2.00 mmol, 5 equiv) in toluene (10 mL) was heated at 100 °C under argon. After 6 h, the solvent was eliminated under reduced pressure and the resulting white solid was dissolved in the minimum volume of acetone (3 mL) and precipitated with diethyl ether (100 mL) five times. The solvents were decanted after each precipitation. The precipitate was than filtered to afford 210 mg of **6** of 96–98% purity (69% yield). ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.73 (quintet, 2H, CH_2), 1.93 (quintet, 2H, CH_2), 2.29 (s, 6H, OAc), 3.67 (t, 2H, CH_2), 4.07 (t, 2H, CH_2), 6.86 (t, 1H, H-4, J = 2.2 Hz), 6.91 (d, 2H, H-2′, H-6′, J = 8.75 Hz), 7.08 (d, 1H, =CH, J = 16.25 Hz), 7.21–7.33 (m, 3H, H-2, H-6, =CH), 7.53 (d, 2H, H-3′, H-5′, J = 8.5 Hz), 7.72–7.96 (m, 15H, aromatic-H). ^{13}C NMR (62.9 MHz, DMSO-d_6) δ (ppm): 19.25 (CH_2), 20.84 (CH_2), 29.25 (CH_2), 65.98 (OCH_2), 114.79, 116.81, 118.46 (Ph, J(^{13}C/^{31}P) = 85.9 Hz), 124.31, 128.06, 129.19, 130.05, 130.25 (Ph, J(^{13}C/^{31}P) = 12.4 Hz), 133.59 (Ph, J(^{13}C/^{31}P) = 10.1 Hz), 134.93 (Ph, J(^{13}C/^{31}P) = 2.9 Hz), 139.76, 151.16, 158.46, 169.01; ESI-MS (ion trap): m/z 629, M^+; HRMS (ESI-TOF): m/z 629.2453; Calcd for $C_{40}H_{38}O_5P^+$ 629.2451.
21. For materials, instrumentation, and experimental details please see Supporting Data.