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Acetal Derivatives as Prodrugs of Resveratrol

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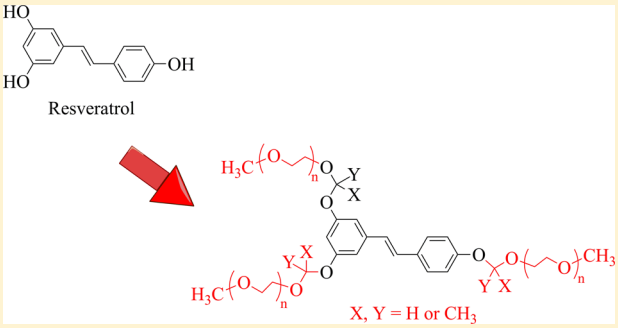
Supporting Information

ABSTRACT: The pharmacological exploitation of resveratrol is hindered by rapid phase-II conjugative metabolism in enterocytes and hepatocytes. One approach to the solution of this problem relies on prodrugs. We report the synthesis and characterization as well as the assessment of *in vivo* absorption and metabolism of a set of prodrugs of resveratrol in which the OH groups are engaged in the formal ($-OCH_2OR$) or the more labile acetal ($-OCH(CH_3)OR$) linkages. As carrier group (R) of the prodrug, we have used short ethyleneglycol oligomers (OEG) capped by a terminal methoxy group: $-O-(CH_2CH_2O)_n-CH_3$ (n = 0, 1, 2, 3, 4, 6). These moieties are expected to exhibit, to a degree, the favorable properties of longer polyethyleneglycol (PEG) chains, while their relatively small size makes for a more favorable drug loading capacity. After administration of formal-based prodrugs to rats by oral gavage, significant concentrations of derivatives were measured in blood samples over several hours, in all cases except for n = 0. Absorption was maximal for n = 4. Complete deprotection to give resveratrol and its metabolites was however too slow to be of practical use. Administration of the acetal prodrug carrying tetrameric OEG chains resulted instead in the protracted presence of resveratrol metabolites in blood, consistent with a progressive regeneration of the parent molecule from the prodrug after its absorption. The results suggest that prodrugs of polyphenols based on the acetal bond and short ethyleneglycol oligomers of homogeneous size may be a convenient tool for the systemic delivery of the unconjugated parent compound.



KEYWORDS: *resveratrol, oligoethyleneglycol, bioavailability, pharmacokinetic, prodrugs*

INTRODUCTION

Resveratrol (Figure 1), one of the most intensely studied plant polyphenols, exhibits a range of activities[1] of biomedical interest. It acts on several cellular signaling pathways,[2−11] overlapping to a considerable extent those mediating the effects of dietary restriction and exercise. Ensuing beneficial effects include lifespan extension in model systems,[12] improvements of functionality in aging,[2,8,10,12,13] protection of the cardiovascular system,[4,14] anti-inflammatory activity,[4] for example in arthritis,[15,16] cancer chemoprevention,[17−19] and potentiation of chemotherapy,[20,21] neuroprotection,[22,23] antagonism of the metabolic syndrome,[24,25] improvement of glucose handling in diabetes,[26] and of fat mobilization.[27,28] Despite the evidence summarized in the cited reviews, the efficacy of resveratrol administration *in vivo* remains in doubt.[29,30] Meta-reviews consider the still preliminary results of human clinical trials inconclusive.[31,32] This is because resveratrol, like other polyphenols, has a relatively poor bioavailability due to a built-in propensity to phase II metabolism, that is, "detoxification" via covalent modification of the hydroxyl groups by the sulfo- and glucuronosyl-transferases of enterocytes and hepatocytes.[33−35] These conjugates can then be re-exported from cells by MDR pumps.[36,37]

Part of the bioefficacy of resveratrol may well be due to hormesis, that is, a response to a low level of stress induced by the bioactive compound, affording protection against subsequent higher doses.[38,39] Such a model implies that even a modest, in absolute terms, increase of bioavailability may have significant consequences, if hormesis induction is achieved. A very relevant aspect currently under investigation is the bioactivity of metabolites formed by phase II "detoxification". The available results suggest that, in general, these conjugates are less bioactive than resveratrol itself,[40−44] hence the dual goal of improving its absorption and of slowing down metabolism to hopefully enhance favorable effects.

Various approaches are being tested: formulations,[45−47] nanovectors,[48,49] and prodrugs[50−53] are the major ones and could represent a useful pharmacological tool to improve the *in*

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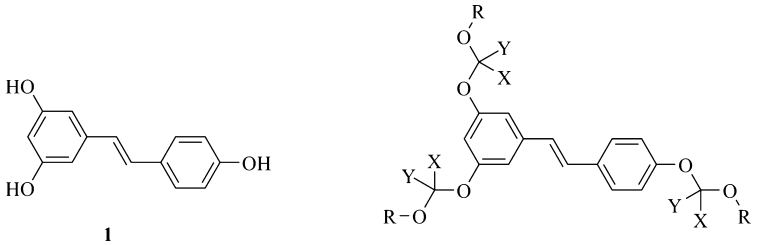

2a-g: X, Y = H;
R = CH_3 (**2a**); CH_2CH_3 (**2b**); $CH_2CH_2OCH_3$ (**2c**); $(CH_2CH_2O)_2CH_3$ (**2d**); $(CH_2CH_2O)_3CH_3$ (**2e**); $(CH_2CH_2O)_4CH_3$ (**2f**); $(CH_2CH_2O)_6CH_3$ (**2g**)
3: X, Y = CH_3; R = CH_3
4: X = H, Y = CH_3; R = $(CH_2CH_2O)_4CH_3$

Figure 1. Resveratrol (**1**) and new derivatives synthesized: formals (**2a−g**), acetals (**3**), and ketals (**4**).

vivo activity of resveratrol.[54] We report here the synthesis, properties, and pharmacokinetics in rats of a family of prodrugs of resveratrol utilizing the acetal bond system to link protective/solubilizing groups to resveratrol hydroxyls ("acetal" is used here, as is customary, as a generic label including all three bonding groups used in this work, see Figure 1). This linkage, often used for the protection of hydroxyls in synthesis procedures, offers the advantage of low polarity and steric hindrance and is therefore expected to allow passage through biomembranes. Furthermore it is acid-sensitive, a characteristic which may lead to a requirement for protection by a suitable formulation during gastric transit, but also offers in principle a tool for targeted release of the active principle in acidic environments. Its use in prodrugs has precedents,[55] and recently a variant has been used to produce derivatives of quercetin, another remarkable polyphenol, with enhanced stability and membrane permeability.[56−58] Notably, the acetal bond system links the polyphenolic scaffold to the glycosidic moiety in polyphenol glycosides, a major class of natural derivatives, and glucuronides, a major product of phase II metabolism. These natural derivatives are characterized by high hydrophilicity and solubility and poor adsorption in their native form.

Polyethyleneglycol (PEG) has been used in a number of formulations and prodrugs to increase solubility, improve absorption, and limit immune response.[59,60] PEG is known to be nontoxic, nonantigenic, and biocompatible, to be rapidly eliminated from the body, to be soluble both in water and many organic solvents, and to have pronounced solubilizing properties. Incorporation of PEG chains can increase resistance to hydrolases and stability in the gastrointestinal tract. PEG has been successfully used as a "carrier" of peptides and proteins as well as of small bioactive molecules. In this latter case however an intrinsic limitation is its low drug loading capacity, that is, the high molecular weight of prodrugs incorporating polymeric chains. We have previously reported the synthesis of a resveratrol prodrug bearing PEG chains linked via carboxyester moieties.[51] In experiments assessing transport across rat intestine, this construct succeeded in determining the translocation of unmodified resveratrol. When using resveratrol as such, practically only conjugation metabolites appeared on the basolateral side. To remedy the low loading capacity we have now used as carrier groups short polyether chains of defined size (oligoethyleneglycol: OEG) and performed a structure−function study to determine the optimal chain size for absorption in pharmacokinetics. To overcome the fragility of the carboxyester moiety[51,61] we have, as mentioned, turned to the acetal linkage.

EXPERIMENTAL SECTION

Materials and General Methods. Resveratrol was purchased from Waseta Int. Trading Co. (Shangai, P.R. China). Other starting materials and reagents were purchased from Aldrich, Fluka, Merck-Novabiochem, Riedel de Haen, J.T. Baker, Cambridge Isotope Laboratories Inc., Acros Organics, Carlo Erba and Prolabo, and were used as received. ^{1}H NMR spectra were recorded with a Bruker AC250F spectrometer operating at 250 MHz and a Bruker AVII500 spectrometer operating at 500 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent. HPLC-UV analyses were performed with an Agilent 1290 Infinity LC System (Agilent Technologies), equipped with binary pump and a diode array detector (190−500 nm). High-performance liquid chromatography/electrospray ionization−mass spectrometry (HPLC/ESI-MS) analyses and mass spectra were performed with a 1100 Series Agilent Technologies system, equipped with binary pump (G1312A) and MSD SL Trap mass spectrometer (G2445D SL) with an ESI source. ESI-MS positive spectra of reaction intermediates and final purified products were obtained from solutions in acetonitrile, eluting with a 1:1 water−acetonitrile mixture containing 0.1% formic acid. Thin-layer chromatographies (TLCs) were run on silica gel supported on plastic (Macherey-Nagel PolygramSIL G/UV_{254}, silica thickness 0.2 mm) or on silica gel supported on glass (Fluka) (silica thickness 0.25 mm, granulometry 60 Å, medium porosity) and visualized by UV detection. Flash chromatography was performed on silica gel (Macherey-Nagel 60, 230−400 mesh granulometry (0.063−0.040 mm)) under air pressure. The solvents were analytical or synthetic grade and were used without further purification. All experiments reported were performed in triplicate unless otherwise stated, and means ± standard deviation values are reported.

*General Procedure for the Preparation of the Formal Derivatives of Resveratrol (**2a−g**).* NaH (17.5 mmol, 4 eq 60% in mineral oil) was washed three times in 5 mL of *n*-hexane. The suspension was decanted after each wash, and hexane traces were removed under reduced pressure. A sample of 10 mL of tetrahydrofuran (THF) was then added, and the suspension was stirred for 5 min. A solution of resveratrol (**1**) (4.4 mmol, 1 equiv) in 5 mL of anhydrous THF was then added. After stirring for 30 min, a solution of the desired chloromethyl ether (24.2 mmol, 5.5 equiv) in 5 mL of anhydrous THF was added dropwise, and the mixture was

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vigorously stirred for 2 min at room temperature. Dimethylformamide (DMF, 5 mL) was then added, and the reaction progress was monitored by TLC. After the disappearance of resveratrol, the reaction mixture was diluted in CH_2Cl_2 (150 mL) and washed with 0.3 N HCl (100 mL). The organic layer was dried over $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by chromatography to give the trisubstituted formal derivative of resveratrol.

*trans-3,4′,5-Tri(methoxymethoxy)stilbene (**2a**).* Purified by flash-chromatography (CH_2Cl_2/AcOEt = 95:5). Yield: 1.15 g (73% based on resveratrol) as a white powder. ESI-MS (ion trap): *m/z* 361 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.49 (s, 3H, −O−CH_3), 3.50 (s, 6H, 2 × −O−CH_3), 5.19 (s, 6H, 3 × −O−CH_2−O−), 6.64 (t, 1H, $^4J_{H,H}$ = 2.3 Hz, H-4), 6.85 (d, 2H, $^4J_{H,H}$ = 2.0 Hz, H-2, H-6), 6.87−7.07 (m, 4H, H-2′, H-6′, H-7, H-8), 7.44 (d, 2H, $^3J_{H,H}$ = 8.8 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.5, 156.9, 139.8, 131.0, 128.8, 127.8, 126.7, 116.4, 107.6, 104.0, 94.5, 94.4, 56.1, 56.0 ppm. Purity (HPLC-UV) ≥ 95%.

*trans-3,4′,5-Tri(ethoxymethoxy)stilbene (**2b**).* Purified by flash-chromatography (hexane/AcOEt = 50:50). Yield: 1.34 g (76%) as a yellow oil. ESI-MS (ion trap): *m/z* 403 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 1.11−1.17 (m, 9H, 3 × CH_2−CH_3), 3.62−3.70 (m, 6H, 3 × −O−CH_2-), 5.24 (s, 6H, 3 × −O−CH_2−O−), 6.57 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.87 (d, 2H, $^4J_{H,H}$ = 2.0 Hz, H-2, H-6), 7.00−7.21 (m, 4H, H-2′, H-6′, H-7, H-8), 7.54 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.0, 156.6, 139.3, 130.3, 128.5, 127.8, 126.3, 116.2, 107.1, 104.5, 92.5, 92.4, 63.7, 63.6, 15.0 ppm. Purity (HPLC-UV) ≥ 95%.

*trans-3,4′,5-Tri-((2-methoxyethoxy)methoxy)stilbene (**2c**).* Purified by flash-chromatography (hexane/AcOEt = 50:50). Yield: 1.44 g (67%) as a yellow oil. ESI-MS (ion trap): *m/z* 493 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.38 (s, 3H, −O−CH_3), 3.39 (s, 6H, 2 × −O−CH_3), 3.56−3.60 (m, 6H, 3 × −O−CH_2−), 3.82−3.86 (m, 6H, 3 × −O−CH_2−), 5.28−5.29 (m, 6H, 3 × −O−CH_2−O−), 6.64 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.87 (d, 2H, $^4J_{H,H}$ = 2,0 Hz, H-2, H-6), 6.93−7.06 (m, 4H, H-2′, H-6′, H-7, H-8), 7.43 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.3, 156.9, 139.6, 130.8, 128.7, 127.6, 126.6, 116.3, 107.5, 104.0, 93.3, 93.2, 71.5, 67.5, 58.9 ppm. Purity (HPLC-UV) ≥ 95%.

*trans-3,4′,5-Tri((2-(2-methoxyethoxy)ethoxy)methoxy)-stilbene (**2d**).* Purified by flash-chromatography (in two steps, using, respectively, CH_2Cl_2/acetone = 80:20 and hexane/AcOEt = 20:85). Yield: 1.84 g (67%) as a yellow oil. ESI-MS (ion trap): *m/z* 625 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.33−3.34 (m, 9H, 3 × −O−CH_3), 3.48−3.52 (m, 6H, 3 × −O−CH_2−), 3.59−3.67 (m, 12H, 6 × −O−CH_2−), 3.82−3.86 (m, 6H, 3 × −O−CH_2−) 5.24−5.25 (m, 6H, 3 × −O−CH_2−O−), 6.61 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.83 −7.04 (m, 6H, H-2, H-6, H-2′, H-6′, H-7, H-8), 7.40 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.3, 156.8, 139.5, 130.8, 128.6, 127.6, 126.5, 116.3, 107.4, 103.9, 93.2, 93.2, 71.7, 70.4, 70.3, 70.2, 67.6, 58.9 ppm. Purity (HPLC-UV) ≥ 99%.

*trans-3,4′,5-Tri(2,5,8,11-tetraoxadodecyloxy)stilbene (**2e**).* Purified by flash-chromatography (hexane/AcOEt/diethyl ether/acetone = 10:30:30:30). Yield: 2.15 g (65%) as a yellow oil. ESI-MS (ion trap): *m/z* 757 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.33−3.34 (m, 9H, 3 × −O−CH_3), 3.47−3.53 (m, 6H, 3 × −O−CH_2−), 3.57−3.66 (m, 24H, 12 × −O−CH_2−), 3.79−3.83 (m, 6H, 3 × −O−CH_2−) 5.23−5.24 (m, 6H, 3 × −O−CH_2−O−), 6.59 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.81 −7.03 (m, 6H, H-2, H-6, H-2′, H-6′, H-7, H-8), 7.39 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.3, 156.9, 139.5, 130.8, 128.6, 127.6, 126.5, 116.3, 107.4, 103.9, 93.2, 93.2, 71.7, 70.4, 70.4, 70.2, 67.6, 58.9 ppm. Purity (HPLC-UV) ≥ 99%.

*trans-3,4′,5-Tri(2,5,8,11,14-pentaoxapentadecyloxy)-stilbene (**2f**).* Purified by preparative HPLC (ACE 5 AQ column 150 × 21.2 mm i.d.). Solvents A and B were H_2O and acetonitrile each containing 0.05% TFA. The gradient for B was as follows: 37.5% for 2 min, from 37.5% to 65% in 10 min. The flow rate was 17 mL/min. The eluate was monitored at 300 nm. Yield: 2.59 g (66%) as a colorless oil. ESI-MS (ion trap): *m/z* 889.5 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.27 (s, 9H, 3 × −O−CH_3), 3.43−3.46 (m, 6H, 3 × −O−CH_2−), 3.52−3.61 (m, 36H, 18 × −O−CH_2−), 3.74−3.77 (m, 6H, 3 × −O−CH_2−) 5.17−5.18 (m, 6H, 3 × −O−CH_2−O−), 6.54 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.76−6.98 (m, 6H, H-2, H-6, H-2′, H-6′, H-7, H-8), 7.34 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.1, 156.7, 139.3, 130.5, 128.4, 127.4, 126.3, 116.1, 107.2, 103.7, 93.0, 92.9, 71.5, 70.1, 70.0, 69.9, 67.4, 58.5 ppm. Purity (HPLC-UV) ≥ 98%.

*trans-3,4′,5-Tri(2,5,8,11,14,17,20-heptaoxahenicosyloxy)-methylenoxy)stilbene (**2g**).* Purified by preparative HPLC (ACE 5 AQ column 150 × 21.2 mm i.d.). Solvents A and B were H_2O and acetonitrile each containing 0.05% TFA. The gradient for B was as follows: from 27.5% to 65% in 13 min. The flow rate was 17 mL/min. The eluate was monitored at 300 nm. Yield: 3.25 g (64%) as a colorless oil. ESI-MS (ion trap): *m/z* 1153.6 $[M + H]^+$. ^1H NMR (250 MHz, $CDCl_3$, 25 °C): δ = 3.21 (s, 9H, 3 × −O−CH_3), 3.47−3.51 (m, 6H, 3 × −O−CH_2−), 3.57−3.64 (m, 60H, 30 × −O−CH_2−), 3.77−3.81 (m, 6H, 3 × −O−CH_2−) 5.22−5.23 (m, 6H, 3 × −O−CH_2−O−), 6.57 (t, 1H, $^4J_{H,H}$ = 2,0 Hz, H-4), 6.79 −7.01 (m, 6H, H-2, H-6, H-2′, H-6′, H-7, H-8), 7.38 (d, 2H, $^3J_{H,H}$ = 8,5 Hz, H-3′, H-5′) ppm. ^{13}C NMR (250 MHz, $CDCl_3$, 25 °C): δ = 158.2, 156.8, 139.5, 130.7, 128.5, 127.5, 126.5, 116.2, 107.3, 103.9, 93.2, 93.1, 71.6, 70.3, 70.3, 70.2, 70.1, 67.5, 58.8 ppm. Purity (HPLC-UV) ≥ 95%.

*trans-3,4′,5-Tri(2-methoxypropan-2-yloxy)stilbene (**3**).* 2-Methoxypropene (1.58 g, 21.9 mmol, 10.0 equiv) was added to a mixture of resveratrol (0.5 g, 2.19 mmol, 1 equiv) and pyridinium *p*-toluenesulphonate (55 mg, 0.22 mmol, 0.1 equiv) under nitrogen pressure and strictly anhydrous conditions. The neat reaction mixture was stirred at room temperature for 72 h. The excess of 2-methoxypropene was removed under reduced pressure, and the crude product was purified by flash chromatography (hexane/acetone = 98:2 + 2% of triethylamine). Yield: 0.20 g (21%) as pale yellow oil. ESI-MS (ion trap): *m/z* 445 $[M + H]^+$. ^1H NMR (300 MHz, acetone-d_6, 25 °C): δ = 1.45 (s, 6H, −O−C(CH_3)$_2$−O−), 1.48 (s, 12H, 2 × −O−C(CH_3)$_2$−O−), 3.38 (s, 3H, −O−CH_3), 3.40 (s, 6H, 2 × −O−CH_3), 6.91 (t, 1H, $^4J_{H,H}$ = 2.1 Hz, H-4), 6.98 (d, 2H, $^4J_{H,H}$ = 2.1 Hz, H-2, H-6), 7.01−7.16 (m, 4H, H-2′, H-6′, H-7, H-8), 7.50 (d, 2H, $^3J_{H,H}$ = 8.7 Hz, H-3′, H-5′) ppm. ^{13}C NMR (300 MHz, acetone-d_6, 25 °C): δ = 157.0, 156.0, 139.7, 132.6, 129.2, 128.2, 127.9, 121.9, 114.2, 113.8, 104.2, 49.2, 25.4, 25.1 ppm. Purity (HPLC-UV) ≥ 95%.

*trans-3,4′,5-Tri(2,5,8,11,14-pentaoxahexadecan-15-yloxy)stilbene (**4**).* Tetraethylene glycol methyl vinyl ether (0.8 g, 3.4 mmol, 6.0 equiv) was added to a mixture of resveratrol

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(0.13 g, 0.57 mmol, 1 equiv) and pyridinium *p*-toluenesulphonate (50 mg, 0.19 mmol, 0.3 equiv) under nitrogen pressure and strictly anhydrous conditions. The neat reaction mixture was stirred at room temperature for 24 h. The crude product was purified by flash chromatography (hexane/acetone = 5:3 + 2% of triethylamine). Yield: 0.23 g (43%) as a pale yellow oil. ESI-MS (ion trap): *m/z* 932 $[M + H]^+$. ^{1}H NMR (500 MHz, acetone-d_6, 25 °C): δ = 1.45−1.47 (m, 9H, 3 × −O−CH(CH_3)−O−, 3.27 (s, 6H, 2 × −O−CH_3), 3.28 (s, 3H, −O−CH_3), 3.44−3.47 (m, 6H, 3 × −O−CH_2−), 3.51−3.62 (m, 36H, 18 × −O−CH_2−), 3.64−3.85 (m, 6H, 3 × −O−CH_2−) 5.52−5.57 (m, 3H, 3 × −O−CH(CH_3)−O−), 6.65 (t, 1H, $^4J_{H,H}$ = 2.0 Hz, H-4), 6.91 (d, 2H, $^4J_{H,H}$ = 1.7 Hz, H-2, H-6), 7.03−7.21 (m, 4H, H-2′, H-6′, H-7, H-8), 7.53 (d, 2H, $^3J_{H,H}$ = 8.6 Hz, H-3′, H-5′) ppm. ^{13}C NMR (500 MHz, acetone-d_6, 25 °C): δ = 159.3, 157.8, 140.6, 131.9, 129.5, 128.6, 127.5, 118.4, 109.6, 106.6, 100.4, 100.4, 72.6, 71.2, 71.2, 71.2, 71.0, 66.0, 66.0, 65.9, 58.8, 20.6, 20.5 ppm. Purity (HPLC-UV) ≥ 95%.

General Procedure for the Preparation of the Non-commercially Available Chloromethyl Ethers. Paraformaldehyde (10.5 mmol, 1.05 equiv) was added to a solution of the desired alcohol (10.0 mmol, 1 equiv) in trimethylsilyl chloride (5 mL), and the reaction mixture was stirred for 2.5 h at room temperature. The solvent was evaporated under reduced pressure, and the chloromethyl ether obtained was used without further purification.

HPLC-UV Analysis. Samples (2 μL) were analyzed using a reversed phase column (Zorbax RRHD Eclipse Plus C18, 1.8 μm, 50 × 2.1 mm i.d.). Solvents A and B were H_2O containing 0.1% TFA and CH_3CN, respectively. The gradient for B was as follows: 10% for 2 min, from 10% to 20% in 3 min, then from 20% to 30% in 1 min, then from 30% to 100% in 1 min; the flow rate was 0.6 mL/min. The eluate was preferentially monitored at 286, 300, and 320 nm. These analytical conditions were used to determine the purity of the synthesized compounds. Formal derivatives **2a**−**g** proved to be stable under these analytical conditions; on the contrary, 0.1% TFA in the eluting aqueous phase (A) caused partial hydrolysis of derivative **4**. For this reason, HPLC analysis of **4** was performed in the absence of TFA. Samples from pharmacokinetic studies of **4** were analyzed both in the absence and in the presence of TFA, to optimally quantify acetal derivatives or resveratrol and phase II metabolites, respectively. Optimal resolution for di- and monosubstituted isomers was achieved using another reversed phase column (Zorbax RRHD Eclipse Plus Phenyl-Hexyl, 1.8 μm, 50 × 2.1 mm i.d.), with a gradient for B as follows: 28% for 3 min, from 28% to 65% in 2 min, then from 65% to 100% in 0.7 min, 100% for 0.5 min.

HPLC/ESI-MS Analysis. Samples (20 μL) were analyzed using a reversed phase column (Synergi-MAX, 4 μm, 150 × 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O containing 0.1% TFA and CH_3CN, respectively. The gradient for B was as follows: 10% for 2 min, from 10% to 35% in 20 min, then from 35% to 100% in 20 min; the flow rate was 1 mL/min. The eluate was preferentially monitored at 286, 300, and 320 nm. MS analysis was performed with an ESI source operating in full-scan mode in positive ion mode.

Solubility in Water. A known amount (approximately 2 × 10^{-5} mol) of the compound was mixed with 2 mL of bidistilled water and kept under stirring in a sealed vial at 25 °C. After 24 h the solution was filtered using a 0.45 μm PTFE filter and centrifuged (10 000 *g*, 10 min, 25 °C). A small aliquot was withdrawn, diluted with acetonitrile, and analyzed by HPLC-UV. Each measurement was repeated in triplicate and also with a mixing time of 48 and 72 h to verify the constancy of the solubility values. The overall average is presented.

Octanol/Water Partition Coefficient (log P_{ow}). Octanol and bidistilled water were reciprocally saturated by shaking them together for 24 h before use. A known amount (approximately 5 × 10^{-7} mol) of the compound was dissolved in 5 mL of octanol and placed in a sealed vial at 25 °C with 5 mL of water under stirring. After 24 h the two phases were separated and centrifuged (12 000 *g*, 20 min). A small aliquot of each phase was then diluted with acetonitrile and subjected to HPLC analysis. Each measurement was repeated in triplicate and also with a mixing time of 48 and 72 h to verify the constancy of the log P_{ow} values. The overall average is presented.

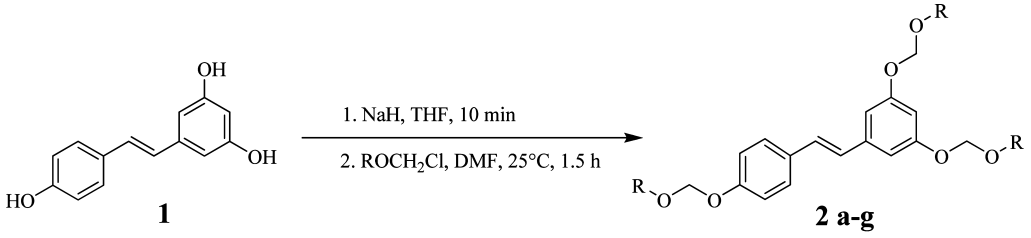
Hydrolysis Reactions. The chemical stability of all new compounds was tested in aqueous media mimicking gastric (0.1 N HCl, NormaFix) and intestinal (0.1 M PBS buffer, pH 6.8) pH values. A 50 μM solution of the compound was made from a 5 mM stock solution in DMSO and incubated at 37 °C for 24 h; samples withdrawn at different times were analyzed by HPLC-UV. Hydrolysis products were identified by HPLC/ESI-MS analysis of selected samples. Nonlinear curve fitting was performed using Origin 8.0 data analysis software, using the equations described in the Supporting Information.

Stability in Blood. Rats were anesthetised and blood was withdrawn from the jugular vein, heparinised and transferred into tubes containing EDTA. Blood samples (1 mL) were spiked with 5 μM of compound (dilution from a 5 mM stock solution in DMSO) and incubated at 37 °C for 4 h (the maximum period allowed by blood stability). Aliquots were taken after 10 min, 30 min, 1 h, 2 h, and 4 h and treated as described below (blood sample treatment and analysis). Cleared blood samples were finally subjected to HPLC-UV analysis. Ad hoc tests demonstrated that compound **4** withstood the extraction procedure without undergoing detectable hydrolysis.

Blood Sample Treatment and Analysis. Before starting the treatment, 4,4′-dihydroxybiphenyl was added as internal standard to a carefully measured blood volume (25 μM final concentration). Blood was then stabilized with a freshly prepared 10 mM solution of ascorbic acid (0.1 vol) and acidified with 0.6 M acetic acid (0.1 vol); after mixing, an excess of acetone (4 vol) was added, followed by sonication (2 min) and centrifugation (12 000 *g*, 7 min, 4 °C). The supernatant was finally collected and stored at −20 °C. Before analysis, acetone was allowed to evaporate at room temperature using a Univapo 150H (UniEquip) vacuum concentrator centrifuge, and up to 40 μL of CH_3CN was added to precipitate residual proteins. After centrifugation (12 000 *g*, 5 min, 4 °C), cleared samples were directly subjected to HPLC-UV analysis. Metabolites and hydrolysis products were identified by HPLC/ESI-MS analysis and/or comparison of chromatographic retention time with true samples.

The recovery yields of resveratrol and its metabolites have been reported previously.[62,63] Internal standard recovery was 68.7 ± 6.3% (N = 7). For the new prodrugs the corresponding recoveries, expressed as the ratio to the recovery of internal standard, were as follows: **2a**: 1.19 ± 0.07; **2b**: 1.18 ± 0.08.; **2c**: 1.21 ± 0.07; **2d**: 0.62 ± 0.06; **2e**: 0.93 ± 0.04; **2f**: 0.77 ± 0.02; **2g**: 0.94 ± 0.05; **4**: 0.69 ± 0.04 (N = 5). Recoveries of partially protected (disubstituted) derivatives were assumed to be the same as those of the corresponding fully substituted prodrug.

Scheme 1. Synthesis of Formal Derivatives (2a–g) from Resveratrol (1) and Alkoxymethylene Chlorides



R = CH_3 (**a**), CH_2CH_3 (**b**), $CH_2CH_2OCH_3$ (**c**), $(CH_2CH_2O)_2CH_3$ (**d**), $(CH_2CH_2O)_3CH_3$ (**e**), $(CH_2CH_2O)_4CH_3$ (**f**), $(CH_2CH_2O)_6CH_3$ (**g**)

Knowledge of these ratios allowed us to determine the unknown amount of analyte in a blood sample by measuring the recovery of the internal standard.

Permeation of the Rat Intestinal Wall (Ex Vivo). Intestine was excised from 18 h fasted rats and transferred into a saline solution (154 mM NaCl in water) at 37 °C. The jejunum was cut into 1 cm long strips, opened longitudinally, rinsed free of luminal content, and mounted in Ussing-type chambers. Apical and basolateral compartments were filled with 1 mL of oxygenated HEPES buffer each (248 mM NaCl, 55.3 mM glucose, 50 mM $NaHCO_3$, 9.9 mM KCl, 1.9 mM $MgSO_4$, 40 mM HEPES, pH 6.8) and incubated in a water bath at 37 °C until all chambers (normally six per experimental run) were assembled. The buffer was then removed and substituted with 1 mL of a 20 μM solution of the compound to be tested in the same buffer on the apical side. During the experiment, oxygen was continuously bubbled in each basolateral compartment. An aliquot of the initial apical solutions was incubated separately at 37 °C for the period of the experiment, to verify the stability of each compound in the absence of jejunum. At the end of the experiment (2.5 h) 800 μL of chamber contents on both apical and basolateral sides were collected and mixed with 8 μL of 100 mM ascorbic acid in water, 8 μL of 6 M acetic acid, and 80 μL of 250 μM 4,4′-dihydroxybiphenyl (internal standard) in CH_3CN. The samples were then centrifuged (12 000 *g*, 7 min, 4 °C), and supernatants were frozen and maintained at −20 °C until HPLC-UV analysis.

Pharmacokinetics. Derivatives **2a–g** and **4** were administered to overnight-fasted male Wistar rats from the stabulary of the Department of Biomedical Sciences as a single intragastric dose (88 μmol/kg, dissolved in 250 μL DMSO). Blood samples were obtained by the tail bleeding technique: before drug administration, rats were anesthetised with isoflurane, and the tip of the tail was cut off; blood samples (80–100 μL each) were then taken from the tail tip at different time points after drug administration. Blood was collected in heparinised tubes, kept in ice, and treated as described below within 10 min. The AUC values were calculated using the trapezoidal rule. All experiments involving animals were performed with the permission and supervision of the University of Padova Ethical Committee for Experimentation on Animals (CEASA) and Central Veterinary Service, in compliance with Italian Law DL 116/92, embodying UE Directive 86/609.

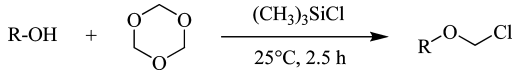
Statistics. Significance in comparisons was assessed using the Wilcoxon Rank Test.

RESULTS

Synthesis. Resveratrol formal derivatives **2a–g** were synthesized via nucleophilic substitution by deprotonated resveratrol (**1**) on the appropriate alkoxymethylene chloride as sketched in Scheme 1. Alkoxymethylene chlorides are well-known and useful reagents for protection of phenolic hydroxyls with formal groups.[64] Despite its apparent simplicity the key step of coupling resveratrol with the alkoxymethylene chloride proved rather tricky. Attempts to perform the reaction in tetrahydrofuran (THF) were not successful: when sodium hydride was added to a THF resveratrol solution, a precipitate was formed, and it did not react appreciably upon addition of alkoxymethylene chloride at room temperature. The desired product was instead obtained in high yield by adding a small amount of dimethylformamide (DMF), which resulted in a very fast change of the suspension color from pale yellow to a dark orange, followed by color fading within a few minutes. It is interesting to note that, when pure DMF was used as solvent, a very fast reaction occurred leading to a complex product mixture, the precipitation of a gummy colored material, and low yields in the desired resveratrol derivative.

The alkoxymethylene chloride agents used for the synthesis of **2a–c** are commercially available, whereas those for **2d–g** had to be synthesized *in situ* from the corresponding alcohol via reaction with paraformaldehyde as shown in Scheme 2. The high reactivity of alkoxymethylene chlorides does not allow their purification via column chromatography on silica. Thus, after removal of excess trimethylchlorosilane under vacuum, they were used immediately in the next step without any further purification.

Scheme 2. Synthesis of Alkoxymethylene Chloride from Alcohol and Paraformaldehyde



The reactivity of the acetal bond can be modulated by the introduction of suitable substituents onto the methylenic carbon. In particular, alkyl groups are known to favor acid-catalyzed hydrolysis.[64] To assess the stability of these bonds in the context of our compounds and conditions we therefore synthesized the ketal **3** and the acetal **4** by reaction of resveratrol with the appropriate isopropenyl and vinyl ethers, respectively, in the presence of catalytic amounts of pyridinium *p*-toluenesulfonate (Scheme 3).

Determination of Solubility and of log P_{ow}. We determined the effect of varying the length of the OEG chain on the solubility in water and on the octanol/water partition coefficient (log P_{ow}) (Table 1) of the formal derivatives. Log P_{ow} is indeed considered to be an important predictive parameter for absorption of passively absorbed drugs.[65]

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Scheme 3. Synthesis of Ketal 3 and Acetal 4

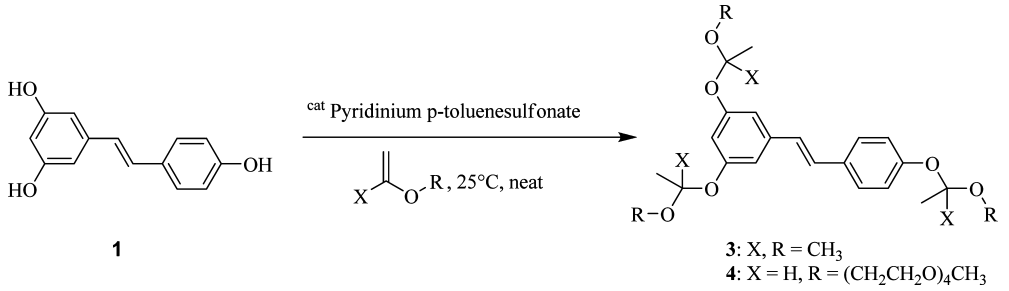

Table 1. Water Solubility and log P_{ow} of Resveratrol and of Its Formal Derivatives (N = 3)

	2a	2b	2c	2d	2e	2f	2g	1
solubility (μM)	*a*	*a*	90 ± 10	230 ± 50	>6000	>6000	>6000	170 ± 40
log P_{ow}	3.41 ± 0.03	3.88 ± 0.09	3.67 ± 0.06	2.95 ± 0.06	2.34 ± 0.01	1.70 ± 0.01	0.43 ± 0.02	2.69 ± 0.12

[a]Below detection limit.

Compounds **2a**–**c** are less soluble than resveratrol itself; the solubility markedly increased with the presence of additional ethoxy groups in the chain. The octanol–water partition coefficients of formal OEG derivatives show an opposite trend. Compounds **2e** and **2f** possess a remarkable solubility in water and have, at the same time, a log P_{ow} value in the optimum range for oral administration and absorption.

Stability Studies. Hydrolysis of **2a** and **2b** was not assayed due to the very low solubility of these compounds in water-based media. The other formals proved stable under near-neutral pH conditions (no reaction over 24 h at 37 °C in 0.1 M phosphate buffer, pH 6.8) as well as in blood (no reaction over 4 h). In 0.1 N HCl they all hydrolyzed at similar rates. For each derivative, suitable HPLC elution conditions were found to separate all components of the hydrolysis reaction mixture comprising, besides the reagent and the final product (resveratrol), four reaction intermediates, that is, the two isomeric disubstituted (3,4′- and 3,5-) and the two isomeric monosubstituted (3- and 4′-) derivatives. As an example, the concentration vs time profiles of all six species involved in the hydrolysis if **2f** are shown in Figure 2a. Kinetic analysis of the data was then performed by assuming that hydrolysis to resveratrol occurs via consecutive losses of the three protecting groups in pseudofirst order processes and by considering each pair of isomeric intermediates as a single species, that is, the two monosubstituted and the two disubstituted intermediates are handled as species B and C, respectively (Scheme 4).

Figure 2b shows the time course of the four species (A, B, C, and D) involved in the case of **2f** and the fit of the experimental data obtained using a set of equations analogous to those utilized by Kozerski et al.[66] and presented as Supporting Information. The excellent fit observed allows to conclude that the assumptions made are justified and that the rate of hydrolysis of each individual formal bond does not depend significantly on the position occupied on the resveratrol scaffold or on the presence of other analogous linkages in the molecule. In fact, at any given time the concentration of one disubstituted resveratrol (the 3,4′-disubstituted) was close to twice that of the other disubstituted isomer (the 3,5-disubstituted), indicating that the formal bonds at the various positions react at about the same rate both in the trisubstituted compound and in the disubstituted ones. Consistently, the amount of one monosubstituted (the 3-substituted) resveratrol was always close to twice that of the other isomer (the 4′-substituted molecule) (Figure 2a).

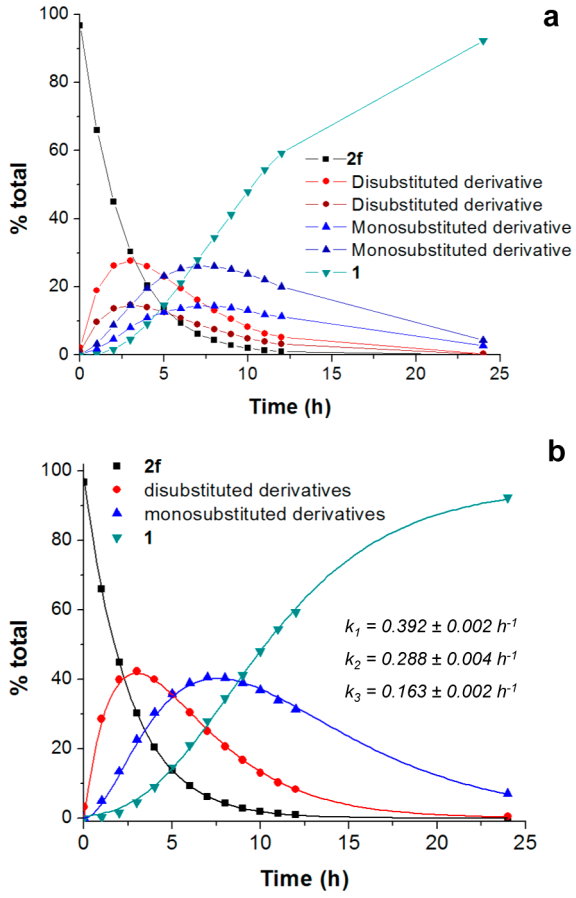

Figure 2. Kinetics of the hydrolysis of **2f** in 0.1 N HCl. Data from a single representative experiment. (a) Time profiles of reagent, product, and four reaction intermediates as obtained by HPLC analysis; (b) kinetic fit of the experimental data shown in panel a according to Scheme 4 (see text and Supporting Information).

Analogous analyses and procedures were used to obtain kinetic data for the hydrolysis of the other formal derivatives (**2c**–**e** and **2g**). The obtained rate constant values are reported in Table S1 (Supporting Information).

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Scheme 4. Kinetic Scheme for the Hydrolysis of 2c–g

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D$$

A: trisubstituted resveratrol derivative

B: disubstituted resveratrol derivatives

C: monosubstituted resveratrol derivatives

D: resveratrol

k_1, k_2, k_3: observed pseudo-first order rate constants

The ketal derivative **3** proved too labile, with about 50% complete hydrolysis to resveratrol in 10 min at pH 7.4 and room temperature (not shown). This compound therefore was not studied further. In contrast, acetal **4** was stable at pH 6.8 and in blood but underwent hydrolysis under acidic conditions. Hydrolysis was too rapid for determination of kinetic parameters at pH 1 (HCl 0.1N). The pH of the rat stomach is however 3–4, higher than in humans.[67] We therefore determined the kinetics of hydrolysis of **4** as a function of pH in a higher pH range (20 mM acetate buffer, pH 3.6–5.6). Figure 3 presents the *k* values, determined with reference to Scheme 4 as described above, for the consecutive hydrolysis steps of **4** in the pH range 4.0–5.6 (a reliable fit could not be obtained for the reaction at pH 3.6).

The results shown in Figure 3 at any given pH value support and extend the conclusions reached for reaction of the formal

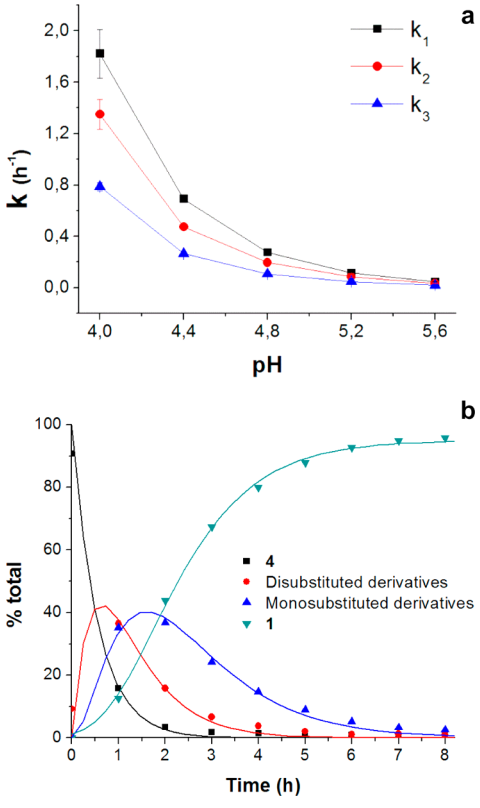

Figure 3. Kinetics of the hydrolysis of **4** at different pH values. (a) Observed rate constants (± standard error) for the hydrolysis of **4**, as a function of pH; (b) kinetic fit of the experimental data at pH 4.0, according to Scheme 4 (see text and Supporting Information).

derivatives described above. It is indeed apparent that the rate of hydrolysis of this class of protecting groups of phenolic hydroxyls is not significantly influenced by the specific site of substitution or by the presence of additional similar groups in the molecule.

Transport across Explanted Rat Intestinal Segments. Using Ussing chambers we also assayed the transport of formals **2a–g** across explanted rat jejunum segments to verify what effect the length of the OEG chain might have on this process. In these experiments, the same molar amount of resveratrol or prodrug was placed in the apical-side chamber, and the solution on the basolateral side was collected and analyzed after 2.5 h (see Materials and Methods for experimental details). Figure 4

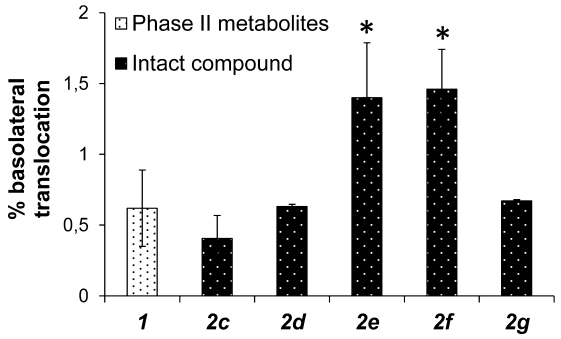

Figure 4. Transport across the explanted rat intestinal segments. Plotted is the amount found in the basolateral chamber (mean ± standard deviation; $N = 3$), expressed as percentage of the total amount of stilbenoid compounds recovered at the end of the 2.5-h incubation. See text and Materials and Methods for details. *: significantly different from basolateral translocation of **1** ($p \leq 0.1$).

summarizes the results, showing the amounts of stilbene derivatives found in the basolateral compartment at the end of the experiment expressed as percentage of the total species recovered in the apical and basolateral sides. When the compound provided was resveratrol, no resveratrol could be detected in the basolateral chamber, which contained instead metabolites adding up to 0.6 ± 0.3% of the total recovered amount. No compounds ascribable to a stilbenoid skeleton could be detected in the basolateral chamber when compounds **2a** or **2b** were loaded. The other formal derivatives appeared instead on the basolateral side without alteration. In the case of compounds **2d** and **2e** the fraction translocated exceeded that of resveratrol (metabolites) (1.4 ± 0.4% and 1.5 ± 0.3% for **2d** and **2e**, respectively; $N = 3$). A substituent chain containing 3 or 4 monomeric units seems therefore optimal for permeation of the intestinal wall.

Pharmacokinetics and Absorption. All synthesized derivatives, except **3**, were then used in pharmacokinetic tests in rats. The administration of 88 μmol/kg of compounds **2a–b** did not result in the appearance of resveratrol, derivatives, or any metabolites in blood samples. Compound **2c** was absorbed to a small extent, but circulating species were below the quantification limit (0.12 μM).[62] In contrast, compounds **2d–f** appeared in blood in unmodified form within 10 min of administration, with a concentration peak between 30 and 60 min. The products of partial hydrolysis, including disubstituted formal derivatives and coeluting trisubstituted species carrying shortened OEG chains, were also found with a similar kinetic profile but in lower concentration. As an example, the results of HPLC/ESI-MS analysis of a blood sample from a pharmaco-

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kinetic experiment with **2f** are shown in Figure 5. Besides a major peak due to **2f**, a few minor and coeluting chromatographic peaks are detected which are assigned to products due to hydrolysis of one formal linkage, to give a disubstituted derivative, and to progressive loss of the terminal methyl groups ($\Delta\ m/z = -14$) and single ethyleneglycol units ($\Delta\ m/z = -44$) (Figure 5). Interestingly, this type of degradation of the substituent groups does not take place upon incubation of the original precursors (**2f** in this case) in solution. We presume these species derive from the action of bacterial enzymes in the gut. Degradation of PEGs by intestinal microbes is known to occur.[68−70] No resveratrol or resveratrol conjugates (glucuronides, sulfates) could be detected in the blood samples with any of the formal-based prodrugs.

The results of pharmacokinetic tests with compound **2f** are summarized in Figure 6, which also shows the pharmacokinetics of resveratrol when administered as such for comparison. Analogous graphs for compounds **2d**, **2e**, and **2g** are provided as Supporting Information (Figure S1).

The outcome of pharmacokinetic experiments with the acetal derivative **4** (Figure 7) was clearly different from those of the tests with the formals. The major species found in blood was resveratrol glucuronide(s) as identified in HPLC/ESI-MS analyses by comparison with an authentic sample. Its concentration peaked at approximately 8 h after administration.

The area under the curve (AUC) parameter for the formal derivatives increases with the length of the OEG chain reaching a maximum for derivative **2f** (Figure 8). Interestingly, in the case of this compound the AUC vastly exceeds that measured for resveratrol as such—and is comparable to the combined value for resveratrol and its metabolites—after administration of the same dose of resveratrol. In the case of **4** the overall AUC was somewhat lower than for **2f**, which carries the same substituent group.

■ DISCUSSION AND CONCLUSIONS

The prodrugs we synthesized and tested possess desirable properties. In the case of acetal derivatives, the protective groups are rapidly hydrolyzed in a strongly acidic environment. In contrast, acid hydrolysis of formals is sufficiently slow, even at pH ∼ 1, similar to that of the human stomach, not to constitute a deterrent: in our pharmacokinetic experiments hydrolysis of formal bond-linked protective groups was indeed very limited. A well-developed formulation technology is available in any case to permit the safe transit of acid-sensitive drugs through the gastric compartment.[71−73] Importantly, short OEG chains appear to be a good choice as carrier group in these resveratrol prodrugs: specifically, for chains comprising three or more monomeric units the derivatives are much more soluble in water than resveratrol. The method we used to determine water solubility led to an estimated value of about 170 μM for resveratrol. It is to note that accurate solubility data are notoriously hard to obtain for compounds which can form colloids, and literature values for resveratrol range from less than 1 μM[74] to about 10 μM[75] and all the way up to 300 μM.[76] Solubility is relevant: essentially water-insoluble **2a** and **2b** do not cross the intestinal wall in Ussing chamber experiments even if kept in solution by the presence of DMSO as a cosolvent and are not absorbed from the gastrointestinal tract after oral administration. OEG-decorated, more soluble derivatives can permeate explanted rat intestinal segments (Figure 4) and are absorbed after oral administration (Figures 6, 7, and 8). The AUC of stilbenoid compounds in blood was

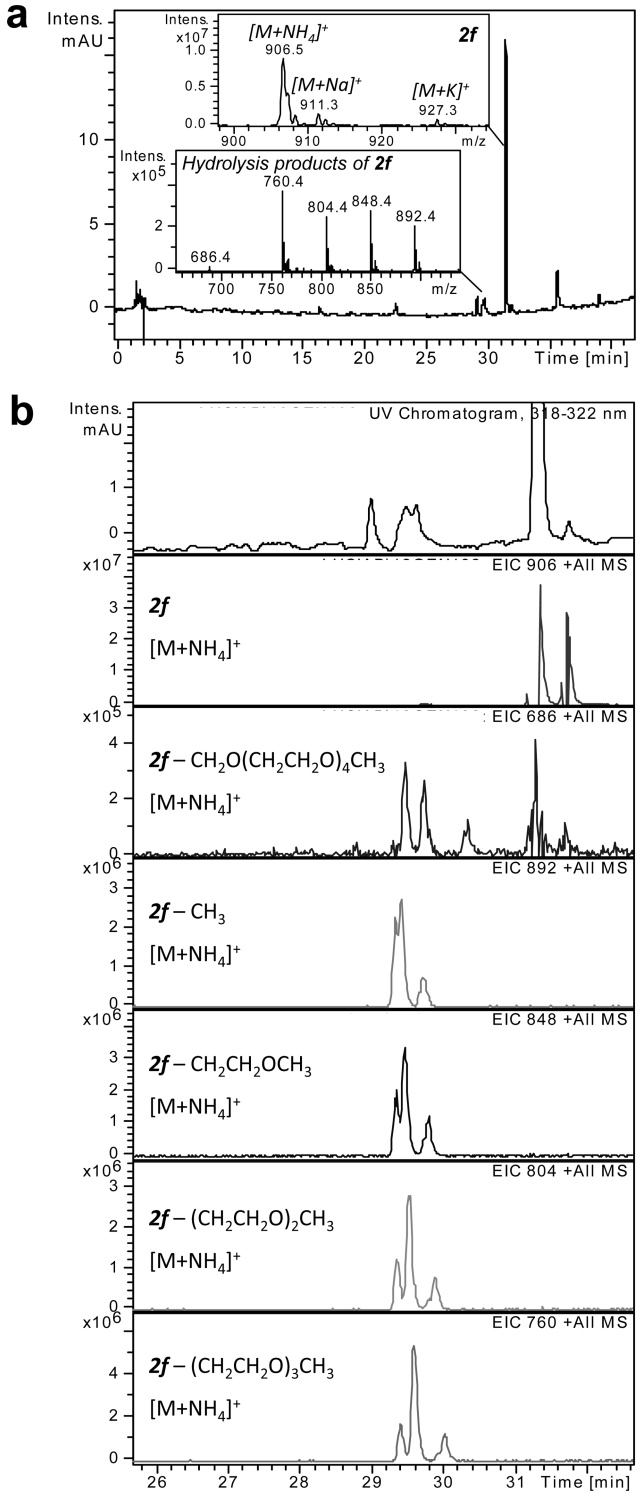

Figure 5. HPLC/ESI-MS analysis of a blood sample taken 1 h after administration of **2f** in a pharmacokinetic experiment. (a) UV chromatogram (320 nm) showing the presence of **2f** and of its partial hydrolysis products (inserts reproduce the corresponding mass spectra). Disubstituted resveratrol derivatives are not well-resolved from the products of partial loss of ethyleneglycol units. (b) Extracted ion chromatograms (EIC) for the m/z values corresponding to intact **2f** (m/z 906 $[M + NH_4]^+$), disubtituted derivatives (m/z 686 $[M + NH_4]^+$) and products of partial loss of ethyleneglycol units (m/z 892, 848, 804, 760, all due to $[M + NH_4]^+$).

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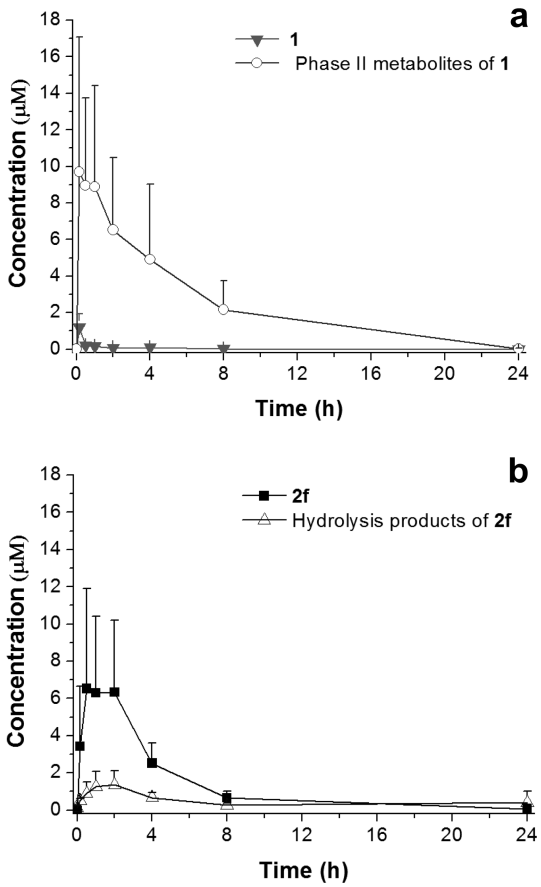

Figure 6. Pharmacokinetic profiles for: (a) resveratrol (**1**) and (b) compound **2f**. "Hydrolysis products" stands for disubstituted derivatives and coeluting species carrying shortened OEG chains as described in the text. Reported are mean values ± standard deviation (N = 5 and 4 for **1** and **2f**, respectively).

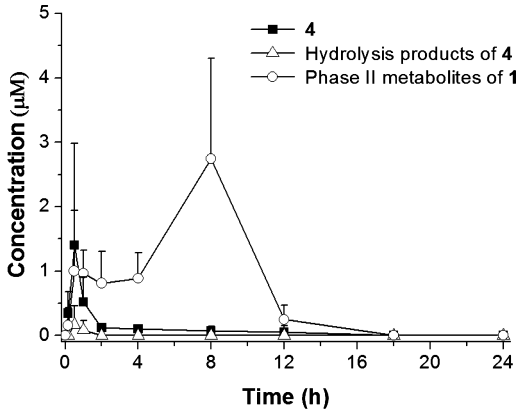

Figure 7. Pharmacokinetics of **4**. Reported are mean values ± standard deviation (N = 4).

similar after administration of resveratrol or of **2f**, the derivative with tetraethyleneglycol chains. This is remarkable when considering the much higher molecular weight and water solubility of the compound and is presumably related to a partition coefficient still favoring permeation of membranes (Table 1). This result, in keeping with the remarkable properties of PEG, offers the perspective of achieving high level systemic or targeted delivery of resveratrol-generating

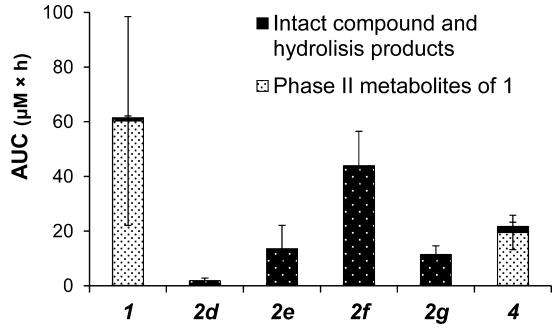

Figure 8. AUC parameter, as calculated from plots analogous to those of Figures 6 and 7. For compounds **2d**–**g** the values reported are the sum of the AUC parameters measured for the prodrug itself and its partial hydrolysis products, which were in all cases a minor component. Conjugation products of resveratrol were detectable only after administration of resveratrol itself (**1**) and of **4**. In these two cases AUC values are shown for both the compound as administered and for total blood stilbenoids.

molecules, bypassing the xenobiotics-inactivating defense systems of the gut and liver.

When resveratrol is administered, the vast majority of the circulating species consists of its phase II metabolites; in the case of our derivatives, still fully or partially protected molecules were found in the bloodstream. This highlights the shortcoming of the formal bond: it is probably too stable at physiological pH values to regenerate the parent compound at useful rates.

To destabilize the bond, we substituted one (**4**) or two (**3**) of the methylene hydrogens with methyl groups. These substitutions had indeed marked effects on the rate of hydrolysis. Compound **3** proved to be too reactive to be of practical use. Compound **4** was also deprotected at an appreciable rate in the pH range of the rat stomach (Figure 3), but deprotection was clearly not complete *in vivo*. The original prodrug and the species resulting from loss of one of the protecting groups were present in blood samples taken over the first hour or so after administration, along with resveratrol glucuronide (Figure 8). At later times, the glucuronide was essentially the only stilbenoid found in blood. Interestingly, its concentration profile in time was different from that observed after administration of resveratrol (Figure 6a): the peak concentration was reached at about 8 h vs about 1 h in the case of resveratrol.

The bond linkage in **4** bears a close resemblance to that present in glycoside and glucuronide derivatives of polyphenols. In the case of glycosides, studies conducted mostly with quercetin derivatives have led to a model envisioning both the cleavage of the acetal bond by lactase phlorizin hydrolase located on the luminal intestinal surface, followed by diffusion of the aglycone into enterocytes, and the transport of the glycoside by glucose transporter SGLT1 followed by deglycosylation inside the cell.[77−80] Similar mechanisms may apply to resveratrol glycosides as well.[81] Considering their log P_{ow} values and what is known about PEG chains-containing prodrugs, our OEG decorated compounds presumably can enter enterocytes and reach the blood by diffusing through biomembranes, independently of transporters. Given that it contains an acetal bond, **4** might be a substrate for lactase phloridzin hydrolase. The results of pharmacokinetics with this prodrug could therefore be explained by slow hydrolysis in the intestine generating resveratrol which would be promptly

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imported by enterocytes and appear in blood as the glucuronide. This model however does not explain why the fully or partially protected species disappear from blood after about one hour: if resveratrol is produced by their hydrolysis in the intestine over the subsequent at least 7 h, they should obviously still be present and continue to transit from the intestinal lumen to blood through the intestinal wall. Of note, we were unable to detect any species containing both OEG chain(s) and glucuronide (or sulfate) moieties in blood. The hypothesis that **4** is completely hydrolyzed in the stomach to produce resveratrol which then moves to the intestine to be absorbed is also unlikely, given the different pharmacokinetics observed upon administration of resveratrol and of **4**.

An alternative model envisions the rapid absorption of the prodrug (as well as of some resveratrol formed in the stomach; Figure 3b), which then disappears from blood because it becomes associated with other, less polar, environments (tissues, membranes) due to its physicochemical properties. Slow hydrolysis, possibly mediated by ubiquitous glucuronidase/glycosylase activities, would gradually release resveratrol which would then be glucuronidated and thus converted to a hydrophilic species, returning therefore to the blood.

Whether this model is correct remains to be investigated. Regardless, this study establishes that linkage to short OEG chains may be a convenient stratagem to protect polyphenols and possibly other drugs from metabolism while still allowing sustained absorption. Coupling such a substituent to the core molecule through a chemical bond with appropriate stability characteristics in physiological environments may well result in a functional prodrug for oral delivery. The acetal bond system, adopted by nature and exploited in this study, appears to be a viable candidate.

■ ASSOCIATED CONTENT

Supporting Information

Kinetics of hydrolysis of compounds **2c−g** in 0.1 M HCl; pharmacokinetic profiles of compounds **2d, 2e, 2g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.M. and M.A. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AcOEt, ethyl acetate; AUC, area under the curve; CEASA, Ethical Committee for Experimentation on Animals; EIC, extracted ion chromatograms; ESI, electrospray ionization; log P_{ow}, octanol/water partition coefficient; MDR, multi-drug resistance; OEG, oligoethyleneglycol; PBS, phosphate-buffered saline; PEG, polyethyleneglycol; PTFE, polytetrafluoroethylene; SGLT1, sodium/glucose cotransporter 1; TFA, trifluoroacetic acid; TLC, thin-layer chromatography

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