Ester-Based Precursors to Increase the Bioavailability of Quercetin

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Plant polyphenols exhibit a variety of potentially useful biochemical properties in vitro, but their evaluation and clinical exploitation in vivo is hampered by their limited bioavailability. Precursors exhibiting resistance to phase II metabolism during absorption are therefore desirable. We report here the synthesis as well as stability and solubility studies of several ester derivatives of quercetin (3,3′,4′,5,7-pentahydroxy flavone), most of which comprise an aminoacyl group. To model transepithelial absorption, we tested transport across supported tight monolayers of MDCK-1, MDCK-2, and Caco-2 cells. Quercetin itself was extensively conjugated by all three types of cells. A few of our precursors did not cross the monolayers, but others did, undergoing partial deacylation. No phase II conjugation was observed during transport of these compounds across MDCK or some Caco-2 clones. With other Caco-2 lines complete deacylation occurred, followed by metabolism of quercetin. Since elimination of residual acyl groups is expected to take place in vivo, ester derivatives of polyphenols may constitute a useful method to increase systemic aglycone concentrations.

1. Introduction

Plant polyphenols, a large and varied class of natural compounds carrying phenolic hydroxyls, exist in a sort of biomedical no man's land. A vast literature, based mostly on in vitro work, describes a variety of potentially important biochemical properties of these compounds. They have long been recognized to act as antioxidants.[1−3] Various abundant members of the family are known to interact with and modulate signal-transducing proteins ranging from channels to cyclooxygenases. Indeed, antioxidant effects are ascribed in part to modulation of transcription factors such as nuclear factor kB and activating protein-1 and to the induction of the expression of antioxidant enzymes such as glutathione-S-transferase and superoxide dismutase.[4,5]

Work with cultured cells, model organisms, and laboratory animals has given support to the notion that various dietary polyphenols—most notably flavones, isoflavones, resveratrol, and epigallocatechingallate (EGCG)[a]—can oppose cancer,[6−9] cardiovascular,[10−12] neurodegenerative,[13,14] and other diseases, and lengthen the lifespan of model organisms[15,16] and people.[17]

Statistical studies, meta-analyses, and human trials, on the other hand, have turned up limited noncontroversial evidence for significant correlations between dietary polyphenol intake and health benefits.[18−22] Even for the best-supported cases, arguably the "French paradox"[23,24] and the low incidence of breast and prostate cancers in soy-consuming populations,[25,26] doubts remain as to the significance of a diet rich in (respectively) resveratrol[27,28] and isoflavones.[29−32]

The discrepancy between in vitro results and epidemiological observations probably has multiple origins. In statistical dietary studies it is notoriously difficult to completely exclude contaminating factors. On the other hand, laboratory results present their own set of problems. Until recently, some studies may have neglected to take into account the fact that the addition of polyphenols to cell culture media may result in the production of hydrogen peroxide,[33,34] i.e., they may act as pro-oxidants (also in vivo, under overload conditions[35]). H_2O_2 is a well-known second messenger, which acts by modifying the activity of signal-transducing proteins, e.g., phosphatases, and can thereby strongly impact cell physiology, e.g., via the activation of MAP kinase cascades.[36] Dosage has been proposed to be a key factor determining the outcome of polyphenol administration,[37] and it has been repeatedly pointed out[38,39] that many in vitro studies have made use of unrealistically high concentrations of the compounds, neglecting the limited bioavailability of dietary polyphenols. This constitutes the major obstacle for a reliable assessment of their effects in vivo as well as, perhaps, for the full exploitation of their pharmacological possibilities. While there are differences depending on the class of compounds considered and on the experimental model used, polyphenols are generally treated by intestinal enterocytes as xenobiotics. According to the current model based on quercetin and resveratrol, the glycosylated derivatives present in food are hydrolyzed to aglycones and glucuronated, sulfated and methylated by detoxifying enzymes after diffusing into the cytosol. These metabolites are mostly re-exported by ABC/MDR-family translocators to the intestinal lumen, where they are eventually degraded by colonic microorganisms, and only a minor fraction is exported to the basolateral side and enters the bloodstream. Analogous conjugation reactions also take place in the liver.[40,41] As a result, only very low levels (in the nanomolar to micromolar range) of polyphenolic compounds are found in blood or plasma even after a polyphenol-rich meal, mostly as metabolites.[42−46] In the case of quercetin, studies with everted intestine segments and analyses of blood and lymph samples have led to the identification of up to 23 different conjugates,[47−53] all arising via single or multiple glucuronation, sulfation, and

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[a] Abbreviations: ABC/MDR, ATP-binding cassette/multiple drug resistance; Boc, *t*-butyl-oxycarbonyl; DMAP, dimethylaminopyridine; EDC, *N*-ethyl-*N*′-(3-dimethylamminopropyl)-carbodiimide; ESI, electrospray ionization; EGCG, epigallocatechingallate; HBSS, Hank's balanced saline solution; MDCK, Madin−Darby canine kidney; PBS, phosphate-buffered saline; PTFE, polytetrafluoroethylene (Teflon); Q, quercetin; TER, transepithelial resistance; TFA, trifluoroacetic acid; TOF, time of flight.

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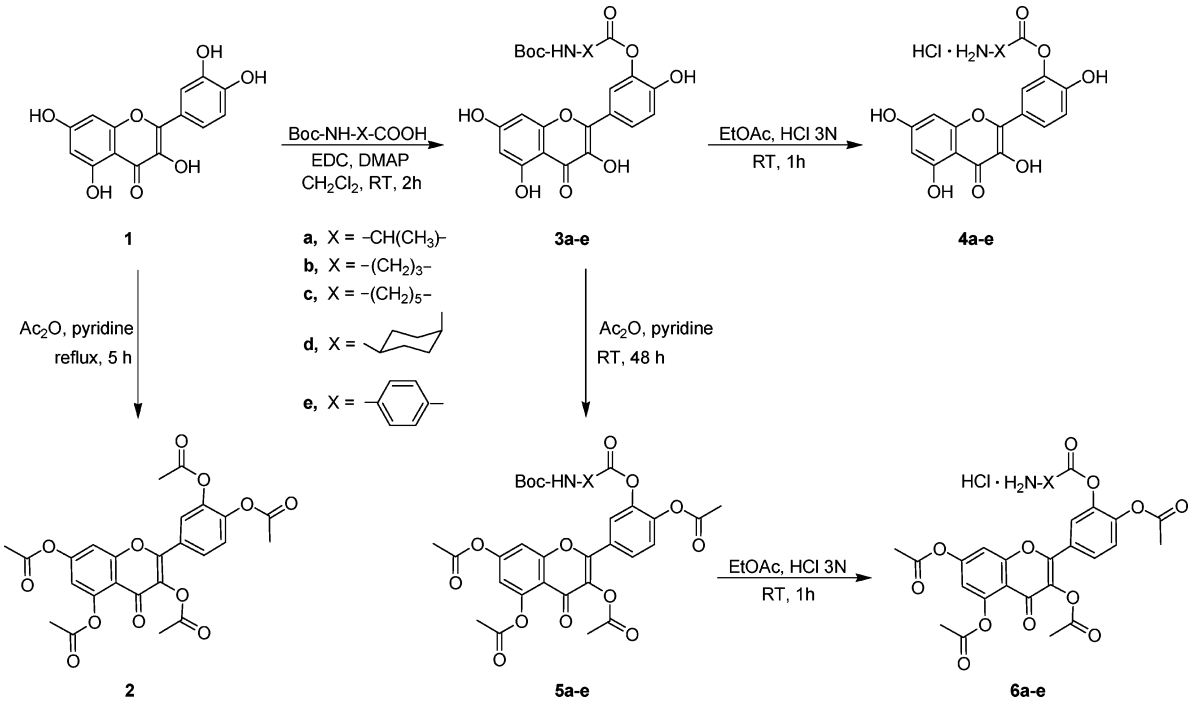
Scheme 1. Synthesis of Quercetin Derivatives



methylation of the hydroxyls. The metabolites may themselves have biological activity.[42,54,55]

In this context we have started work aiming at bypassing the modification of polyphenols by enterocytic transferases. This would improve bioavailability, increasing the levels of the aglycone in the circulatory stream and organs. In turn, this would allow more reliable assessments of the efficacy and innocuity of the compounds under various experimental conditions mimicking circumstances of (patho)physiological relevance and might eventually lead to the development of polyphenol-based molecules with preventive or curative applications. One way the bypass may be achieved is via the development of polyphenol precursors capable of withstanding transit through epithelia and of regenerating the parent compound once in circulation. This paper reports initial progress toward such goals.

Given the nature of the molecules in question, we focused on ester derivatives, also because the ubiquitous presence of enzymes with esterase activity[56−58] promises the rapid regeneration of the parent polyphenol in vivo.

Bioavailability- and/or stability-improving ester prodrugs have already been developed for many drugs with alcoholic or phenolic hydroxyls.[59,60] In the case of polyphenols, peracetylated EGCG[61,62] and quercetin,[63−66] and water-soluble 3′-(*N*-carboxymethyl)carbamoyl-3,4′,5,7-tetrahydroxyflavone (QC12)[67] have been reported. However, the effects of these modifications on transepithelial transport and metabolism have not been investigated.

Since this is intended as a proof-of principle study, we concentrated on a model polyphenol, choosing quercetin (3,3′,4′,5,7-pentahydroxy flavone, **1**) because it is abundant in nature, readily available, present in plasma almost totally as conjugates, and because its absorption and metabolism have been studied in detail.[47−53,68−72] To evaluate the effects of derivatization on transepithelial transport, we employed supported cell monolayers of three different epithelial cell lines, namely, canine MDCK-1 and -2 and human Caco-2. The latter are often used as a stand-in for intestinal epithelium. This experimental setup has been utilized in two studies of the transport of quercetin and its glucosides across Caco-2 monolayers. One[73] reported a rapid passage of unmodified quercetin. The other[74] found conjugation products on both sides.

We produced a series of quercetin derivatives bearing an aminoacyl substituent to promote solubility and permeation. The recent literature contains several reports of amino acid-based prodrugs. Transport of the aminoacyl esters across Caco-2 monolayers has been evaluated in a few of these investigations, and found to be improved in comparison with that of the parent drug in some cases.[75−77] Resistance to hydrolysis was reported to depend on the aminoacyl group.[78] Enhanced resistance to metabolism was also observed.[75,79]

We thus report in this paper the synthesis, characterization, and the hydrolytic and cell monolayer permeation properties of a series of quercetin esters. Most comprised an aminoacyl functionality, which we varied seeking to optimize the properties of the compounds.

2. Results

2.1. Synthesis. Scheme 1 outlines the synthesis of compounds **2**−**6**. Pentaacetylquercetin (**2**) was synthesized according to a published procedure.[63] Attempts to obtain pentaaminoacylquercetins via reaction of quercetin with excess NH_2-blocked amino acids yielded intractable mixtures of polysubstitution products. We therefore proceeded to synthesize monoaminoacyl derivatives. The reaction of Boc-protected amino acids with an excess of quercetin (**1**) resulted in esterification mainly of the B ring hydroxyls, in line with the acidity,[80] high oxidability, and reactivity of the catechol moiety. Compounds **3a**−**e** were isolated by flash chromatography and turned out to be a mixture of 3′- and 4′-substituted quercetins, in an approximately 3:1 ratio. The two isomers would be expected to have very similar properties, and in fact our strenuous attempts to separate them by chromatography were unsuccessful. Therefore, all compounds mentioned in this work were actually a mixture of two isomers. NMR characterization led to the assignment of 3′ as the major esterification site: compared to the corresponding protons in quercetin, $\delta_{H2'}$ is greater than $\delta_{H5'}$ in the major product, while the opposite is true for the minor product (not shown).

Table 1. Observed Rate Constants for the Hydrolysis of 3′-Aminoacylquercetins in 1:1 PBS/CH_3CN[a]

derivative	k_{obs} (s^{-1})	$t_{1/2} \times 10^{-2}$ (s)
3a	$(6.56 \pm 0.04) \times 10^{-4}$	10.7
3b	$(9.3 \pm 0.2) \times 10^{-5}$	75
3c	$(5.18 \pm 0.03) \times 10^{-5}$	132
3d	$(5.8 \pm 0.7) \times 10^{-5}$	119
4a	$(2.16 \pm 0.03) \times 10^{-2}$	0.32
4b	$(9.7 \pm 0.3) \times 10^{-2}$	0.07
4c	$(4.2 \pm 0.2) \times 10^{-4}$	16.5
4d	$(7.4 \pm 0.6) \times 10^{-4}$	9.3

[a] Error notations are average deviations ($N = 2$ or 3).

The remaining hydroxyls were acetylated with acetic anhydride in pyridine at room temperature (compounds **5a**−**e**). The Boc group was removed by treating compounds **3a**−**e** and **5a**−**e** with EtOAc/HCl to afford NH_2-free amino acid esters (compounds **4a**−**e** and **6a**−**e**).

2.2. Chemical Stability Studies. Assessing the stability of the compounds in aqueous solution was important both for transepithelial transport experiments and, a priori, to distinguish esterase-mediated and spontaneous hydrolysis. Hence, the chemical stability of **2**−**6** was evaluated by monitoring their UV−vis spectra in a mixture of PBS (20 mM, pH 7, $\mu = 0.1$ M)/CH_3CN 1:1, where CH_3CN was necessary to ensure the complete solubilization of all the compounds.

Compounds **2**, **3e**, **4e**, **5a**−**e**, and **6e** turned out to be stable under these conditions. All the other compounds underwent hydrolysis. Compounds **3a**−**d** and **4a**−**d** hydrolyzed to quercetin, as shown by the comparison of the final UV−vis spectrum of the reaction mixture with that of a 1:1 mix of **1** and the amino acid. The kinetic constants and estimated half-life times ($t_{1/2}$), obtained from exponential fits of plots of $A_{255.2}$ versus time, are listed in Table 1.

NH_2-free amino acid esters **4a**−**d** hydrolyzed significantly faster than NH_2-protected amino acid esters **3a**−**d**. Furthermore, the presence of the Boc-group affected stability ranking of the amino acid esters. Among Boc-protected esters the least stable was the D-alanine derivative ($t_{1/2}$: **3a** < **3b** < **3d** < **3c** < **3e**), while γ-aminobutanoic acid turned out to be the most labile substituent for esters bearing a free $-NH_2$ group ($t_{1/2}$: **4b** < **4a** < **4d** < **4c** < **4e**). 4-Aminobenzoic acid derivatives were the most stable ones in both cases.

Compounds **6a**−**d** were also unstable in the presence of PBS buffer. They underwent two consecutive hydrolytic processes, as evidenced by the appearance of two isosbestic points in the spectra recorded in the 190−500 nm range. Figure 1 illustrates a representative experiment. MS spectra of products and HPLC−UV and LC−MS analyses (not shown) led to the conclusion that the amino acid substituent was lost first, followed by an acetyl group.

A subsequent hydrolysis of the resultant triacetylquercetin was also observed at longer reaction times, but it did not influence the interpolation of the data (see the Experimental Section), as no substantial variation in absorbance was associated with this process. The kinetic constants of the first two hydrolytic processes are reported in Table 2. In all cases, hydrolysis could be blocked by acidification. Addition of 0.2% formic acid halted the progression of spectral changes (and allowed analysis of the solution). As can be appreciated from Tables 1 and 2, the stability of the compounds having free −OH and/or $-NH_2$ groups was inadequate for their utilization in transport experiments. Accordingly, they were not investigated further.

2.3. Solubility in HBSS. In Table 3 the approximate solubility in HBSS is reported for the compounds found to be stable in aqueous solution at near-neutral pH. Solubilities were in the same range as for quercetin, except in the cases of *cis*-4-aminocyclohexanecarboxylic acid and 4-aminobenzoic acid derivatives, for which they were below 1 μM. It should be pointed out that the values reported represent upper limits: part of the material in "solution" was presumably still present as colloidal particles rather than as a true solute.

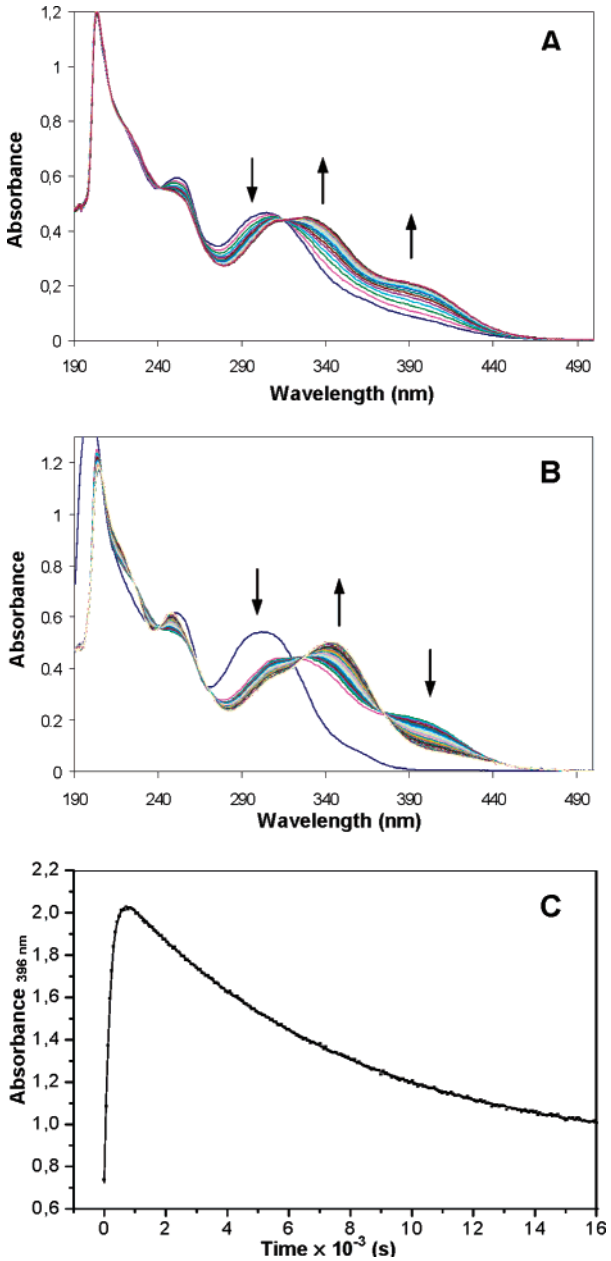


Figure 1. Hydrolysis of **6a** in PBS/CH_3CN 1:1. (A) UV−vis spectra recorded every minute for 30 min. (B) UV−vis spectra recorded every 10 min for 8 h. (C) Interpolation of the data with eq 2.

Table 2. Observed Rate Constants for the First and Second Hydrolysis of 3′-Amino Acid Tetraacetylquercetin Ester Hydrochlorides in 1:1 PBS/CH_3CN[a]

derivative	$k_{1,obs}$ (s^{-1})	$k_{2,obs}$ (s^{-1})	$t_{1/2} \times 10^{-2}$ (s)
6a	$(5.03 \pm 0.26) \times 10^{-3}$	$(1.26 \pm 0.07) \times 10^{-4}$	1.37
6b	n.d.	$(1.33 \pm 0.10) \times 10^{-4}$	n.d.
6c	$(9.6 \pm 0.9) \times 10^{-4}$	$(1.32 \pm 0.18) \times 10^{-4}$	0.72
6d	$(2.51 \pm 0.07) \times 10^{-4}$	$(1.23 \pm 0.07) \times 10^{-4}$	27.6

[a] Error notations are average deviations ($N = 2$ or 3). The tabulated $t_{1/2}$ of the compounds refers to the first process.

Table 3. Approximate Solubilities in HBSS of Stable Compounds at Room Temperature

compound	solubility in HBSS (μM)
1	2.3
2	1.1
3e	0.4
4e	1.5
5a	12.0
5b	2.5
5c	3.9
5d	0.8
5e	0.4
6e	1.0

2.4. Transepithelial Transport. Due to their short lifetime in water solutions at pH values compatible with cell function, or to the presence of unprotected hydroxyls, compounds **3a**−**e**, **4a**−**e**, and **6a**−**d** were considered unsuitable for transepithelial transport experiments. Experiments were therefore performed only with compounds **1**, **2**, **5a**−**e**, and **6e**. Quercetin (**1**) obviously served as benchmark. To better characterize polyphenol metabolism, which depends on cell type, three different popular cellular models were employed, namely, MDCK-1, MDCK-2, and Caco-2, which form monolayers upon differentiation. A total of 223 individual transport experiments were carried out. The extent of transport depended on the size of the Transwell insert used, since the surface/volume ratio is different in the two cases (see the Experimental Section). The composition of the product mixtures in both compartments changed with time, as translocation, hydrolysis, and conjugation progressed. Analyses of the basolateral compartment from representative experiments with 24-mm supports, at 6 h, are reported in Table 4. Mass balances (i.e., the sum of the molar amounts of all compounds present in all compartments at a given time over the moles of quercetin derivative placed in the apical compartment at time zero) were in the range of 70−85% after 3 h, declining to 50−70% after 6 h. They were similar for transport experiments and for control incubations without cells. Approximately 20% of the material originally loaded in the apical compartment was found in association with the cell layer, from which it could be extracted with methanol (see the Experimental Section). The composition of this fraction was intermediate between those of the apical and basolateral compartments.

2.4.1. Quercetin (1). The "diffusion" of quercetin from the apical to the basolateral compartment was characterized to define the metabolic activities of the cells and as a reference for the performance of its derivatives. As expected, only a small percentage of **1** appeared as such in the basolateral compartment (Table 4). After 6 h, and using 24-mm inserts, this amounted to approximately 1%, 3%, and 1% of the quantity loaded in the apical compartment in the case of MDCK-1, MDCK-2, and Caco-2 cells, respectively. Quercetin sulfate, methylquercetin (probably isorhamnetin, i.e., 3′-methoxyquercetin[74]), methylquercetin sulfate, and quercetin *o*-quinone were the other compounds detected on the basolateral side. Figure 2A presents typical HPLC−UV_{370} chromatograms from basolateral samples taken after 6 h. The *o*-quinone is not detected at the wavelength used in the figure (its peak absorbance is at 295 nm). Transport kinetics are illustrated by the set of chromatograms in Figure 2B. The identity of the compounds was established by comparison of retention times with standards (quercetin, 4′-methylquercetin), UV−vis spectral characteristics (quercetin sulfate,[41] quercetin *o*-quinone[81]), mass spectrometry, and comparison of the chromatographic profiles of untreated and sulfatase-treated samples (Figure 3). The formation of quercetin glucuronides by Caco-2 cells has been reported in the literature.[82] In our hands they could be detected, at low levels, in a few experiments (including the one in Figure 2), but more often they were below detection limits.

Table 4. Percentages of the Intact Compounds and their Metabolites in the Basolateral Compartment after 6-h Transport Experiments Across MDCK-1, MDCK-2, and Caco-2 Monolayers, using 24-mm Inserts[a]

		% of apically added material at 6 h		
compound loaded	basolateral species	MDCK-1	MDCK-2	Caco-2
1	intact compound (Q)	0.9	2.7	1.0
	Q-sulfate + methyl-sulfate	6.6	0.6	5.8
	methyl-Q	0.9	0.8	1.3
	Q-*o*-quinone	0.3	1.7	0.8
	total basolateral	8.7	5.8	8.9
2	intact compound		0.5	
	tetraacetyl-Q	0.4	0.9	
	diacetyl-Q	8.4	12.7	
	monoacetyl-Q	1.0	4.7	
	Q	1.6	2.5	0.4
	Q-sulfate + methyl-sulfate			5.0
	methyl-Q			0.5
	Q-*o*-quinone	0.6	1.2	0.5
	total basolateral	12.0	22.5	6.4
5a	intact compound			
	diacetyl-Q	2.4	8.2	
	monoacetyl-Q	2.2	2.4	
	Q	0.6	0.8	4.2
	Q-sulfate + methyl-sulfate			1.8
	methyl-Q			2.4
	Q-*o*-quinone	1.1	2.6	2.6
	total basolateral	6.3	14.0	11.0
5b	intact compound			
	diacetyl-Q	4.6	6.5	
	monoacetyl-Q	1.2	3.2	0.4
	Q	0.3	1.0	2.6
	Q-sulfate + methyl-sulfate			2.2
	methyl-Q			0.9
	Q-*o*-quinone	1.4	3.2	2.8
	total basolateral	7.5	13.9	8.9
5c	intact compound			
	diacetyl-Q	13.6	5.6	
	monoacetyl-Q	6.5	3.9	0.3
	Q	2.1	2.6	2.5
	Q-sulfate + methyl-sulfate			3.4
	methyl-Q			2.0
	Q-*o*-quinone	4.3	3.1	2.9
	total basolateral	22.2	12.1	8.2

[a] Representative experiments are reported. Figures given are percentages of the total amount of starting material present in the apical compartment at time zero.

MDCK-1 and Caco-2 cells on one side and MDCK-2 cells on the other exhibited reproducible differences, in that the latter appeared to possess weaker sulfate- and methyl-transferase activities so that the major specie found on the basolateral side was quercetin itself. Caco-2 cells turned out to be complex. In the course of this work we used seven lines, which were outwardly very similar but handled quercetin and its derivatives differently. "Clone A" (actually two lines) exhibited strong phase II conjugation activity; the basolateral mixture of products obtained was similar to that of MDCK-1 cells. Five other lines, which we refer to as "clone B" for simplicity, had only weak methyl- and sulfotransferase activities, behaving much like MDCK-2 cells. These observations will be reported in detail elsewhere. Since in vivo experiments indicate that quercetin is heavily conjugated during and/or after absorption, clone A may be considered to best reflect in vivo processes. All Caco-2-related results presented in this paper were obtained with these cells.

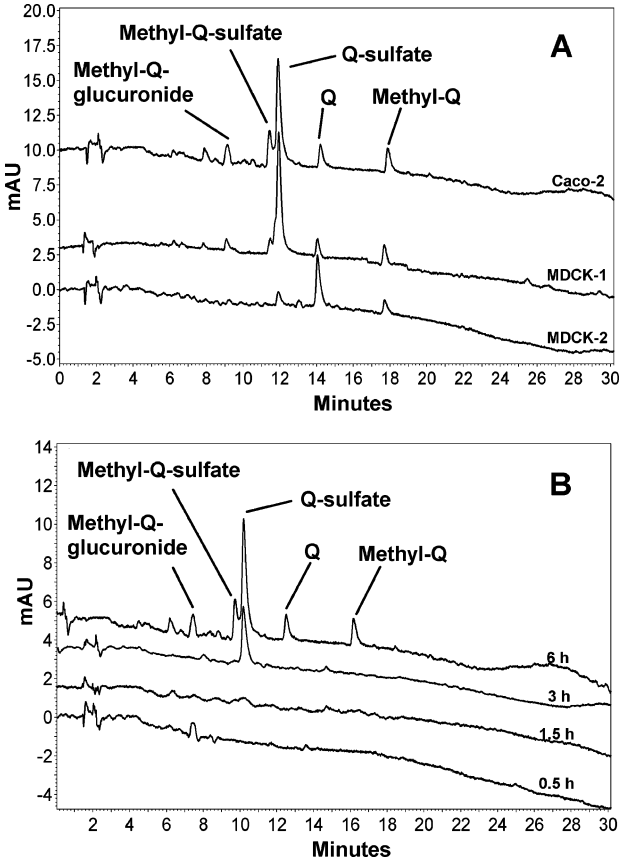


Figure 2. HPLC−UV chromatograms (370 nm) of the basolateral side in transport experiments with quercetin (**1**) (24-mm inserts): (A) after 6 h, with the three types of cell monolayers; (B) with Caco-2 cells at the indicated times.

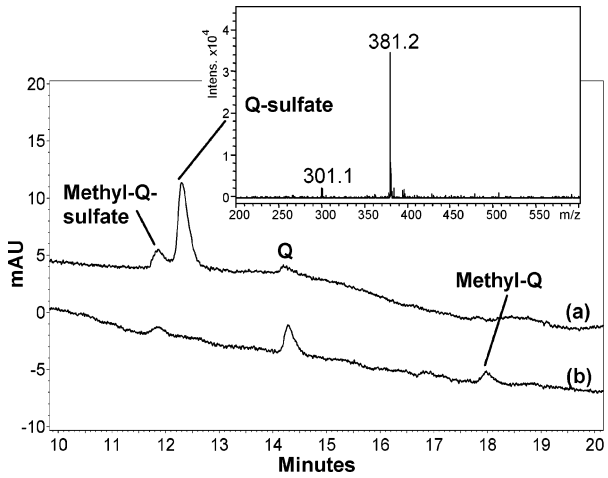


Figure 3. HPLC−UV chromatograms (370 nm) of a basolateral side sample taken after 5 h in an experiment of quercetin transport across a Caco-2 monolayer (12-mm insert), before (a) and after (b) treatment with sulfatase. Inset: negative ESI-MS spectrum of quercetin sulfate.

The composition of the apical compartment changed relatively slowly when the canine cell lines were employed. Quercetin was the major compound remaining up to at least 6 h. Minor amounts of the same metabolites found in the basolateral compartment appeared, along with some (2−3% at 6 h) quercetin *o*-quinone. With Caco-2 cells apical quercetin underwent more drastic modification, to give the usual conjugation products (Figure 4). These findings are in broad agreement with literature reports.[74,82]

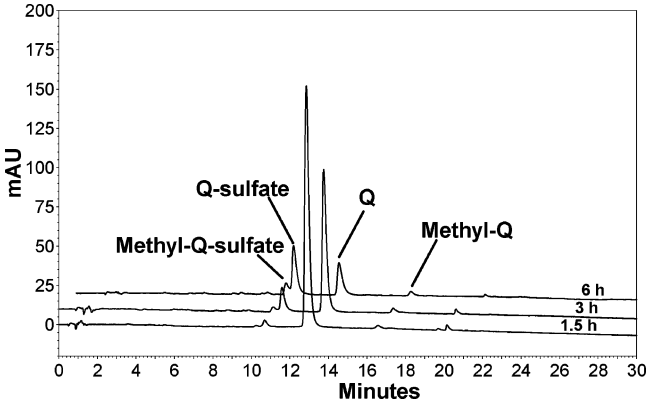


Figure 4. HPLC−UV chromatograms (370 nm) of the apical side in experiments of quercetin transport across Caco-2 monolayers using 24-mm inserts. The upper two chromatograms have been shifted to the right for clarity.

2.4.2. Pentaacetylquercetin (2). The fraction of apically loaded pentaacetylquercetin that reached the opposite compartment without undergoing alterations was also very low for all three cell lines (Table 4). With this compound, as well as the others mentioned below, a major difference between MDCK-1 and MDCK-2 cells on one side and Caco-2 cells on the other was evident. The former partially deacetylated **2** but did not generate detectable amounts of sulfated derivatives in any compartment. The latter efficiently stripped off all acyl groups, and proceeded to rapidly conjugate the aglycone, so that the major products were the same compounds observed when the transport substrate was quercetin itself (Figure 5).

The composition of the apical compartment was qualitatively similar to that of the corresponding basolateral one, except for the obvious presence of **2** itself, which declined in time becoming a relatively minor component after 6 h. With MDCK-1 and -2 cells, acetylquercetins and some quercetin were the only detectable products. Interestingly, only small quantities of tetraacetylquercetins and even lesser amounts of triacetylquercetins (at least three different isomers, when detectable) formed in either the apical or basolateral compartments, while the diacetyl specie was prominent (not shown; see Figure 7).

With Caco-2 cells, some quercetin, larger percentages of sulfated and/or methylated quercetin than in the case of MDCK monolayers, and small amounts of partially acetylated quercetins formed in the apical compartment (not shown).

2.4.3. 3′-Boc-amino Acid Tetraacetylquercetins 5a−c. The behavior of compounds **5a**−**c** was similar to that of **2**. Again, there was a marked difference between Caco-2 cells and the other lines. With both MDCK and "clone B" Caco-2 lines, acylated forms but no parent or sulfated compounds appeared in the basolateral compartment (Table 4 and Figure 6). The partially acetylated forms consisted mainly of diacetyl- and monoacetylquercetin, with very minor quantities of Boc-amino acid triacetylquercetin ester(s) at intermediate times and no detectable tri- and tetraacetylquercetin(s). With "clone A" Caco-2 monolayers, acylated forms were conspicuously absent, except for very small amounts of monacetylquercetin. Sulfated and/or methylated quercetins, and quercetin itself, were the only derivatives present in quantifiable amounts (Table 4, Figure 6). With any given cell type, the chromatographic profile was qualitatively the same for the three compounds (**5a**−**c**).

In the apical compartment the starting compound was progressively hydrolyzed, with the loss of most of it after 6 h. Interestingly, in all cases the reaction taking place most readily was not the loss of the Boc-aminoacyl substituent, but rather

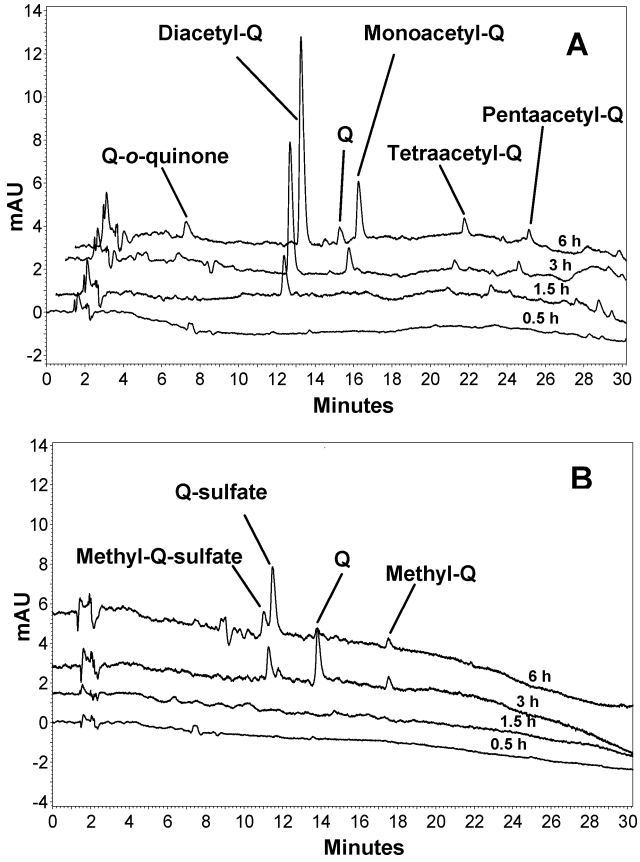


Figure 5. HPLC−UV chromatograms of the basolateral side in experiments of compound **2** transport across (A) MDCK-2 monolayers (300 nm) and (B) Caco-2 monolayers (370 nm), using 24-mm inserts. In (A) the upper three chromatograms have been shifted to the right for clarity.

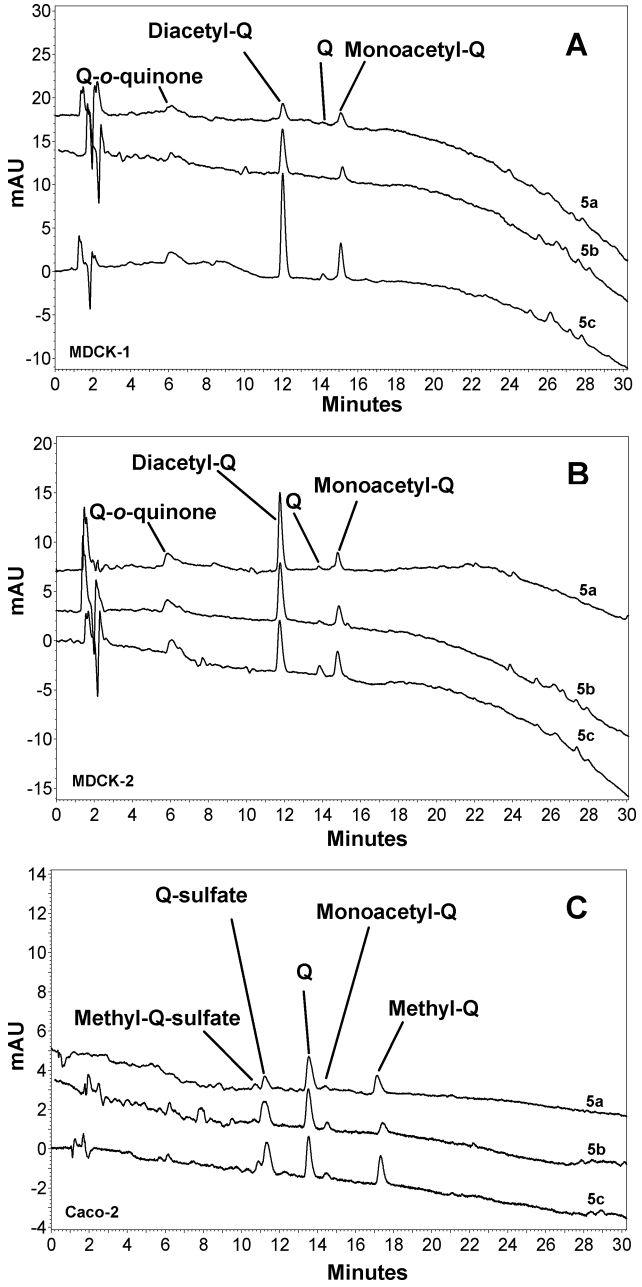


Figure 6. Comparison between HPLC−UV chromatograms of the basolateral side after 6 h in transport experiments of compounds **5a**−**c** across (A) MDCK-1 (300 nm), (B) MDCK-2 (300 nm), and (C) Caco-2 monolayers (370 nm).

that of an acetyl group, leading to the accumulation of significant amounts of Boc-amino acid triacetylquercetin ester. Again, MDCK cells did not generate detectable amounts of sulfated derivatives, while Caco-2 cells did (Figure 7).

2.4.4. 3′-Boc-amino Acid Tetraacetylquercetins 5d,e and Compound 6e. With these compounds no detectable transepithelial transport took place over 6 h. Little happened in the apical compartment as well. The only derivatives detected, in trace amounts, were partially deacylated compounds (not shown).

3. Discussion

The goal of the project is the development of bioavailability-enhancing precursors of polyphenols. In this phase we have taken the most straightforward approach, synthesizing first-generation ester derivatives of a model compound, quercetin, and testing them on in vitro epithelial models. While obtaining compounds simultaneously permeant, sufficiently soluble, and stable in aqueous media proved difficult, some of the molecules we tested yielded encouraging results.

The test tube chemistry of quercetin is dominated by that of its catechol group, and it is not surprising that the monoester derivatives were formed at either the 3′ or the 4′ position. The 3:1 predominance of substitution at 3′ may reflect thermodynamic control: the molecule with a free −OH in 4′ may well be slightly more stable since its conjugate base can delocalize the negative charge onto the oxygen at position 4.

The presence of a free −OH in ortho to the ester group in the catecholic moiety contributes to the instability of 3′- and 4′-acylquercetins in aqueous solution. This is an example of the "ortho effect", a case of general base catalysis: the deprotonated phenolic hydroxyl can aid the nucleophilic attack of a molecule of water to the neighboring carbonyl,[83] thus accelerating the decomposition of monosubstituted compounds (**3a**−**d**, **4a**−**d**). With persubstituted derivatives, once the first acyl (acetyl or aminoacyl) substituent is lost from a catecholic position the neighboring one is also condemned. This two-stage process could be clearly followed spectroscopically in the case of compounds **6a**−**d** (Figure 1). That an ionized group is involved follows from the fact that hydrolysis of monosubstituted compounds, as well as the second stage of the two-phase process undergone by **6a**−**d**, was blocked by acid. This feature helped us to identify the products of the first step. Unfortunately, the pH values required to stop decomposition were not compatible with cell welfare. The "ortho effect" explains the second stage of the biphasic hydrolysis of peresterified quercetins, but a primary loss of the aminoacyl group preceded it, unless the

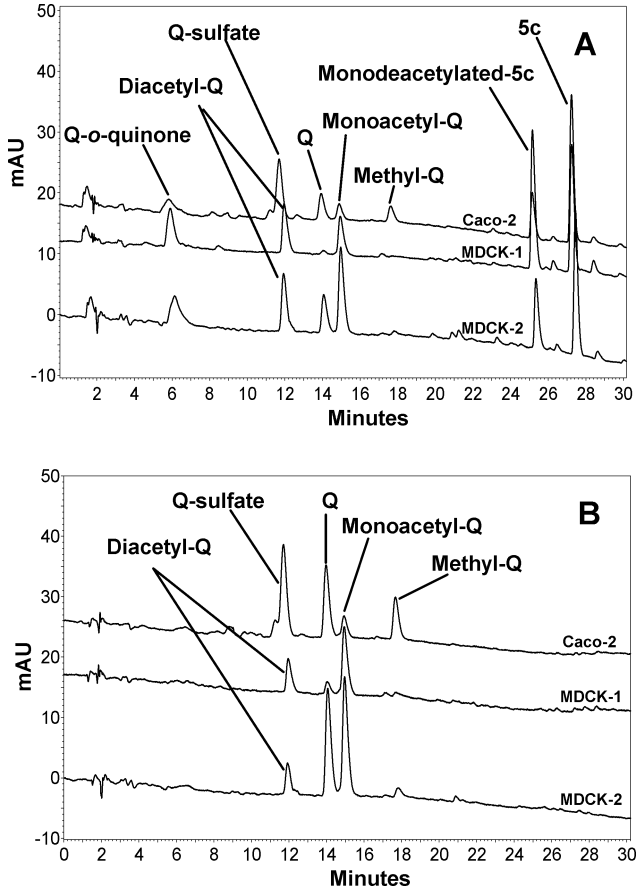


Figure 7. Comparison between HPLC−UV chromatograms of the apical side after 6 h in experiments of **5c** transport across Caco-2, MDCK-1, and MDCK-2 monolayers, recorded at (A) 300 nm and (B) 370 nm. Note that the peak of diacetylquercetin appears lower at 370 nm while those of quercetin and quercetin sulfate appear higher at this wavelength.

$-NH_2$ group was blocked by the *t*-butyl-*O*-carbonyl group. The destabilizing effect of the amino group probably has multiple mechanistic explanations, having different relevance for the various compounds. If close to the acyl carbonyl, as in **4a** and **6a**, the amino group, especially if protonated ($-NH_3^+$), is expected to exert a "through-bond" electron-withdrawing effect, increasing the polarization of the carbonyl and therefore its susceptibility to nucleophilic attack. The Boc group, besides protecting for steric reasons, would be expected to decrease the inductive effect because its presence turns the amino group into a less basic, and less electron-withdrawing, amido group. In fact, the Boc-protected compounds (containing a free ortho −OH) hydrolyze less rapidly than the unprotected ones (compare the kinetics of **3a** and **4a**). A polarization effect can take place also "through space", thus accounting perhaps in part for the instability of compounds in which the acyl chain can fold in such a way as to bring the amino group into proximity with the acyl carbonyl, as in compounds **4c** or **6c**. Straightforward intermolecular base catalysis is however likely to be more important. In fact, in the case of **6d**, in which such a folding is unlikely, and the $-NH_2$ is too far from the carbonyl for a "through-bond" effect to be plausible, the rate of hydrolysis remains too high for the compound to be useful for our purposes. When also this last prohydrolyis catalytic option is removed, lowering the basicity of the $-NH_2$ group by turning it into an anilino group in compound **6e**, stability is finally achieved. But the solubility of **4e** and **6e**, even in the absence of the hydrophobic Boc group, is very low.

Deacylation of the water-stable compounds in the presence of cells also showed some interesting features. Triacetylquercetin, the expected product of deacylation of the two catecholic positions, was never detected in more than trace amounts, while di- and monoacetylquercetins were relatively abundant. The first process taking place was the loss of an acetyl group, yielding tetrasubstituted quercetin (Figure 7). The group lost was most likely on the A or C rings, since departure of the acetyl on the B ring would be expected to precipitate the rapid loss of the neighboring acyl group, producing trisubstituted species. Indeed, as soon as one of the B ring esters is hydrolyzed, the neighboring one follows suit, resulting in the formation of diacetylquercetin. In Caco-2 (but not MDCK) cells the two remaining ester groups of the condensed ring system are also rapidly hydrolyzed, before conjugation takes place, since we did not detect any acetylquercetin sulfate or (acetyl)-methylquercetin.

The compounds used in transport experiments did not undergo spontaneous (as distinct from cell-mediated) hydrolysis at an appreciable rate, but their stability was undermined by other processes, largely responsible for lowering the mass balance of our experiments. The amount of material used up in this manner tended to vary, suggesting that erratic processes, possibly oxidative chain reactions, occurred. No products plausibly arising from processes of this type could be detected in either transport experiment samples or in ad hoc solutions/suspensions allowed to age under air. They presumably consist of polymeric materials retained by our LC columns. Remarkably, this "loss" of material took place in water but not when the compounds were dissolved in organic solvents. A clue to what may be happening is offered by studies of the oxidation chemistry of quercetin and other polyphenols.[1,81,84−86]

The complications discussed above hindered the development of an "ideal" quercetin precursor. Nonetheless, the transport experiments were promising. They highlight the fact that different cells (even ostensibly belonging to the same line) can treat polyphenol derivatives differently. All three reference lines we used handled quercetin much like intestinal enterocytes, allowing almost only conjugated derivatives to cross the monolayer. The same products were found in cellular extracts and progressively formed in the apical compartment, confirming the existence of two-way traffic between the cellular interior and the apical extracellular space. The observation that different Caco-2 lines exhibit different levels of conjugation activities may explain discrepancies between observations by different groups.[73,74,82]

The fate of the various derivatives depended on cell type. "Clone A" Caco-2 cells proved to be the best equipped with relevant hydrolase and transferase activities. Apparently, the esters which could diffuse into the cells were rapidly stripped of protective groups and the resulting quercetin was processed. The other lines were less efficient, sufficiently so that partially deacylated molecules were the major products in the basolateral compartment. The changes in the composition of the apical compartment were consistent with this pattern. Since most of the material found in basolateral samples had been metabolized, the transport process must have involved intracellular (as opposed to paracellular) pathways. Some compounds (**5d**, **5e**, **6e**) survived for at least 6 h in the presence of the cells with little change and did not cross the monolayers in detectable amounts. This resilience may be related to their particularly low solubility in aqueous media.

The total molar fraction of permeating quercetin derivatives reaching the basolateral side was not significantly higher than

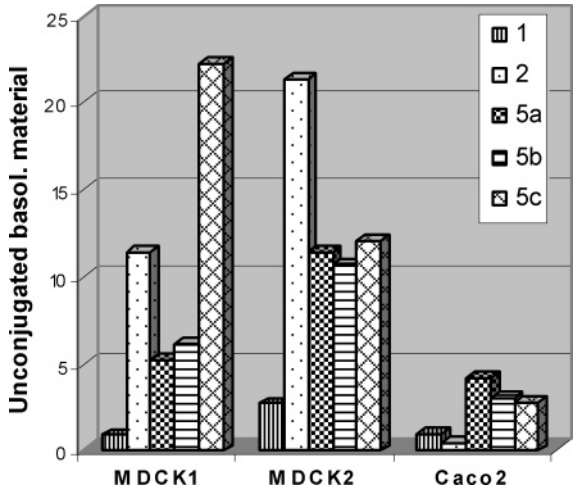


Figure 8. Use of precursors increases the portion of quercetin reaching the basolateral compartment without being conjugated. The histograms report, for the three cell lines, the molar percentage of the compounds placed in the apical compartment at time zero found to have translocated to the basolateral side after 6 h, without undergoing sulfation, methylation, or glucuronidation. Data from Table 4.

that of quercetin itself (Table 4), but in vivo the acylated species—which in favorable cases make up most of the basolateral material—would be expected to be rapidly converted to quercetin by esterases so that the net result would be an increase in the fraction of quercetin absorbed as such. This point is illustrated by Figure 8, which compares the total basolateral amounts of unconjugated compounds for the various precursors and cell lines. Esterases are found both inside and outside cells in practically all organs (especially in epithelia).[56−58] Although carboxylesterase EC 3.1.1.1 may not be present in human (as distinct from mammalian) blood,[87] the esterase activity of albumin,[87,88] which binds quercetin,[89,90] may well be sufficient for quercetin regeneration (Biasutto, L., et al., unpublished results).

A point of obvious relevance is the relationship between these observations in vitro and what may happen in vivo. Clarification of this point can only come from suitable experiments. Since Caco-2 cells are human, while MDCKs are canine (and renal), the results obtained with the former may seem more likely to predict the eventual outcome of these experiments. However, as already mentioned, several other Caco-2 clones afforded results similar to those obtained with MDCK-2 cells. Furthermore, absorption of polyphenols in the intestine, the prevalent site,[91] may or may not resemble processes handled by colonic tumoral cells. Caco-2 carboxyesterases are actually expressed in a pattern more resembling that of liver than that of the intestine.[92] For research and hypothetical treatment purposes, absorption via less aggressive epithelia may in any case be an option.

In conclusion, our results show that it may be feasible to develop ester-based precursors of naturally occurring polyphenols, capable of improving absorption through the intestinal tract or other less aggressive epithelia. In vivo tests and the development of a second generation of compounds are in the making.

4. Experimental Section

4.1. Chemistry. 4.1.1. General. Starting materials and reagents were purchased from Aldrich, Fluka, Merck-Novabiochem, J. T. Baker, Cambridge Isotope Laboratories Inc., Acros Organics, Carlo Erba, and Prolabo and were used as received. ^{1}H NMR spectra were recorded on a Bruker AC 250F spectrometer operating at 250 MHz. Chemical shifts (δ) are given in ppm relative to the signal of the solvent (δ 2.49 ppm, DMSO-d_6). Mass spectra were recorded on a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems) or an ESI MSD SL Trap mass spectrometer (Agilent Technologies). 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. HPLC analyses were performed by a Thermo Separation Products Inc. system with a P2000 Spectra System pump and a UV6000LP diode array detector (190−500 nm). Elemental analyses, HPLC−UV, and solubility determinations were done only for the compounds used for transport experiments (**2**, **5a**−**e**, **6e**). Elemental analyses were performed by the Microanalysis Laboratory of the Department of Chemical Sciences of the University of Padova. HPLC−UV was carried out as described in the transport experiments section.

4.1.2. Synthesis of Pentaacetylquercetin (2). Compound **2** was synthesized in 67% yield from **1**, according to a literature procedure.[63] Briefly, **1** (3.021 g, 8.93 mmol) in acetic anhydride (30 mL) and pyridine (2.7 mL) were heated to reflux for 5 h. Ice-water (50 g) was added to the warm mixture. The resulting precipitate was filtered and washed with water. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 2.32 (s, 3H, 3-OAc), 2.34 (s, 12H, aromatic-OAc), 7.16 (d, 1H, H-6, J = 2.2 Hz), 7.52 (d, 1H, H-5′, J = 8.6 Hz), 7.64 (d, 1H, H-8, J = 2.2 Hz), 7.35−7.65 (m, 2H, H-2′, H-6′). MS (ESI-TOF): m/z 513, $[M + H]^+$.

4.1.3. General Synthetic Procedure of Amino Acid Quercetin Esters. 4.1.3.1. 3′-Boc-amino Acid Quercetin Ester (3a−e). Boc-protected amino acids (Boc-D-alanine, 4-(Boc-amino)butanoic acid, 6-(Boc-amino)hexanoic acid, *cis*-4-(Boc-amino)cyclohexanecarboxylic acid, 4-(Boc-amino)benzoic acid) (5 mmol) were dissolved in CH_2Cl_2 (10 mL). DMAP (1 mmol) and EDC (5 mmol) were added, and the solution was stirred. Compound **1** (7.5 mmol), dissolved in DMF (5 mL), was added. The mixture was stirred for 1 h at room temperature. CH_2Cl_2 was evaporated under reduced pressure, and the mixture was dissolved in EtOAc (700 mL). The resulting solution was washed successively with 0.5 N HCl (3 × 100 mL), water (2 × 70 mL), 5% $NaHCO_3$ (3 × 100 mL), and water (2 × 70 mL), dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography to afford the 3′-substituted Boc-amino acid ester of quercetin containing 25% of the 4′-isomer, as determined by NMR analysis. The two isomers were not separated. Reported yields include both isomers; NMR data refer to the major one.

4.1.3.2. 3′-Amino Acid Quercetin Ester Hydrochloride (4a−e). The 3′-Boc-amino acid quercetin ester (0.1 mmol) was dissolved in 3.5 mL of a 3 N HCl solution in EtOAc. The mixture was stirred for 1 h at room temperature. The resulting precipitate was filtered and washed with diethyl ether.

4.1.3.3. 3′-Boc-amino Acid Tetraacetylquercetin (5a−e). The 3′-Boc-amino acid quercetin ester (0.6 mmol) was dissolved in CH_2Cl_2 (12 mL). Acetic anhydride (13.70 mmol) and pyridine (1.2 mL) were added, and the mixture was stirred for 5 h at room temperature. CH_2Cl_2 was evaporated under reduced pressure, and the mixture was dissolved in EtOAc (20 mL) and washed with 0.5 N HCl (3 × 7 mL), water (2 × 5 mL), 5% $NaHCO_3$ (3 × 7 mL), and water (2 × 5 mL). The EtOAc solution was dried over Na_2SO_4 and filtered. EtOAc was evaporated under reduced pressure to afford the acetylation products.

4.1.3.4. 3′-Amino Acid Tetraacetylquercetin Hydrochloride (6a−e). 3′-Boc-amino acid tetraacetylquercetin (0.078 mmol) was dissolved in a solution of 3 N HCl in EtOAc (3 mL). The mixture was stirred at room temperature for 1 h. The resulting precipitate was filtered and washed with diethyl ether.

3′-Boc-D-alanine Quercetin Ester (3a). Eluents of flash chromatography: CH_2Cl_2/EtOAc 3:1. Yield: 28%. ^{1}H NMR (250 MHz,

DMSO-d_6) δ (ppm): 1.38−1.48 (s, 12H, CH_3), 4.32 (br, 1H, CH), 6.18 (d, 1H, J = 2.2 Hz), 6.41 (d, 1H, J = 2.2 Hz), 7.07 (d, 1H, H-5′, J = 8.7 Hz), 7.41 (br, 1H, NH), 7.81 (d, 1H, H-2′, J = 2.2 Hz), 7.95 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): *m/z* 474 $[M + H]^+$, *m/z* 947 $[M + H]^+$(M), *m/z* 418 $[MH-CH_2{=}C(CH_3)_2]^+$.

3′-D-Alanine Quercetin Ester Hydrochloride (4a). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.60 (d, 3H, CH_3), 4.45 (q, 1H, CH), 6.21 (d, 1H, J = 2.2 Hz), 6.46 (d, 1H, J = 2.2 Hz), 7.17 (d, 1H, H-5′, J = 8.2 Hz), 8.08 (m, 2H, H-2′, H-6′), 8.64 (br, $^+NH_3$). MS (ESI-TOF): *m/z* 374 $[M + H]^+$.

3′-Boc-D-alanine Tetraacetylquercetin Ester (5a). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.30−1.70 (m, 12H, CH_3), 2.25−2.65 (m, 12H, OAc), 4.30 (m, 1H, CH), 7.16 (d, 1H, J = 2.2 Hz), 7.54 (d, 1H, H-5′, J = 8.7 Hz), 7.59 (br, 1H, NH), 7.64 (d, 1H, J = 2.2 Hz), 7.75 (d, 1H, H-2′, J = 2.2 Hz), 7.87 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): *m/z* 642 $[M + H]^+$, *m/z* 586 $[MH-CH_2{=}C(CH_3)_2]^+$.

3′-D-Alanine Tetraacetylquercetin Ester Hydrochloride (6a). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.57 (d, 3H, CH_3), 2.21−2.41 (m, 12H, OAc), 4.51 (br, 1H, CH), 7.18 (d, 1H, J = 2.2 Hz), 7.60 (d, 1H, H-5′, J = 8.7 Hz), 7.62 (d, 1H, J = 2.2 Hz), 7.86 (d, 1H, H-2′, J = 2.2 Hz), 7.93 (dd, 1H, H-6′, J = 8.7, 2.2 Hz), 8.59 (br, $^+NH_3$). MS (ESI-TOF): *m/z* 542 $[M + H]^+$, *m/z* 500 $[MH- CH_2CO]^+$.

3′-[4-(Boc-amino)butanoic Acid] Quercetin Ester (3b). Eluents of flash chromatography: $CHCl_3/CH_3OH$ 9:1. Yield: 16%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.38 (s, 9H, CH_3), 1.74 (quintet, 2H, CH_2-β), 2.60 (t, 2H, CH_2-α), 3.03 (q, 2H, CH_2-γ), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.91 (t, 1H, NH), 7.06 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2 Hz), 7.95 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): *m/z* 488 $[M + H]^+$, *m/z* 432 $[MH-CH_2{=}C(CH_3)_2]^+$, *m/z* 975 $[M + H]^+$(M).

3′-[4-Aminobutanoic Acid] Quercetin Ester Hydrochloride (4b). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.89 (quintet, 2H, CH_2-β), 2.76 (t, 2H, CH_2-α), 2.88 (br, 2H, CH_2-γ), 6.21 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 7.14 (d, 1H, H-5′, J = 8.7 Hz), 7.87 (d, 1H, H-2′, J = 2.2 Hz), 7.94 (dd, 1H, H-6′, J = 8.7, 2.2 Hz), 7.93 (br, $^+NH_3$). MS (ESI-TOF): *m/z* 388 $[M + H]^+$.

3′-[4-(Boc-amino)butanoic Acid] Tetraacetylquercetin Ester (5b). Yield: 77%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH_3), 1.74 (quintet, 2H, CH_2-β), 2.31 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.62 (t, 2H, CH_2-α), 3.01 (q, 2H, CH_2-γ), 6.92 (t, 1H, NH), 7.16 (d, 1H, J = 2.2 Hz), 7.52 (d, 1H, H-5′, J = 9.0 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.76−7.93 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): *m/z* 678 $[M + Na]^+$, *m/z* 600 $[MH-CH_2{=}C(CH_3)_2]^+$.

3′-[4-Aminobutanoic Acid] Tetraacetylquercetin Ester Hydrochloride (6b). Yield: 84%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.94 (quintet, 2H, CH_2-β), 2.29 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.78 (t, 2H, CH_2-α), 2.87 (br, 2H, CH_2-γ), 7.17 (d, 1H, J = 2.2 Hz), 7.53 (d, 1H, H-5′, J = 8.7 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.78−8.02 (br, $^+NH_3$), 7.81−7.91 (m, 2H, H-2′, H-6′). MS (ESI-TOF): *m/z* 556 $[M + H]^+$.

3′-[6-(Boc-amino)hexanoic Acid] Quercetin Ester (3c). Eluents of flash chromatography: $CHCl_3/CH_3OH$ 9:1. Yield: 24%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.36 (s, 9H, CH_3), 1.30−1.53 (m, 4H, CH_2), 1.63 (quintet, 2H, CH_2), 2.59 (t, 2H, CH_2), 2.85−2.98 (m, 2H, CH_2), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.78 (t, 1H, NH), 7.06 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2 Hz), 7.96 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): *m/z* 516 $[M + H]^+$, *m/z* 460 $[MH-CH_2{=}C(CH_3)_2]^+$, *m/z* 1031 $[M + H]^+$(M).

3′-[6-Aminohexanoic Acid] Quercetin Ester Hydrochloride (4c). Yield: 79%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.34−1.52 (m, 2H, CH_2), 1.52−1.78 (m, 4H, CH_2), 2.62 (t, 2H, CH_2), 2.78 (sextet, 2H, CH_2), 6.21 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 7.11 (d, 1H, H-5′, J = 8.7 Hz), 7.85 (d, 1H, H-2′, J = 2.2 Hz), 7.88 (br, $^+NH_3$), 7.93 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-TOF): *m/z* 416 $[M + H]^+$.

3′-[6-(Boc-amino)hexanoic Acid] Tetraacetylquercetin Ester (5c). Yield: 68%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.36 (s, 9H, CH_3), 1.27−1.53 (m, 4H, CH_2), 1.63 (quintet, 2H, CH_2), 2.31 (s, 3H, 3-OAc), 2.33 (s, 9H, aromatic-OAc), 2.62 (t, 2H, CH_2), 2.85−2.97 (m, 2H, CH_2), 6.79 (br t, 1H, NH), 7.16 (d, 1H, J = 2.2 Hz), 7.52 (d, 1H, H-5′, J = 8.2 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.77−7.91 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): *m/z* 706 $[M + Na]^+$, *m/z* 722 $[M + K]^+$.

3′-[6-Aminohexanoic Acid] Tetraacetylquercetin Ester Hydrochloride (6c). Yield: 82%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.31−1.49 (m, 2H, CH_2), 1.49−1.74 (m, 4H, CH_2), 2.33 (s, 12H, OAc), 2.65 (t, 2H, CH_2), 2.69−2.82 (m, 2H, CH_2), 7.17 (br, 1H), 7.53 (br d, 1H, H-5′, J = 8.2 Hz), 7.64 (br, 1H), 7.73−7.91 (br, 5H, H-2′, H-6′, $^+NH_3$). MS (ESI-TOF): *m/z* 584 $[M + H]^+$.

3′-[*cis*-4-(Boc-amino)cyclohexanecarboxylic Acid] Quercetin Ester (3d). Eluents of flash chromatography: $CHCl_3/CH_3OH$ 9:1. Yield: 22%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH_3), 1.47−1.77 (m, 6H, CH_2), 1.90−2.12 (m, 2H, CH_2), 2.80 (br, 1H, CH), 3.41 (br, 1H, CH), 6.19 (d, 1H, J = 2.2 Hz), 6.45 (d, 1H, J = 2.2 Hz), 6.83 (br d, 1H, NH), 7.07 (d, 1H, H-5′, J = 8.7 Hz), 7.81 (d, 1H, H-2′, J = 2.2 Hz), 7.94 (dd, 1H, H-6′, J = 8.7, 2.2 Hz). MS (ESI-ion trap): *m/z* 550 $[M + Na]^+$.

3′-[*cis*-4-Amino-cyclohexanecarboxylic Acid] Quercetin Ester Hydrochloride (4d). Yield: 76%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.58−1.96 (m, 6H, CH_2), 2.04−2.28 (m, 2H, CH_2), 2.96 (br, 1H, CH), 3.15 (br, 1H, CH), 7.17 (d, 1H, H-5′, J = 8.7 Hz), 7.78−7.97 (br, 5H, H-2′, H-6′, $^+NH_3$). MS (ESI-ion trap): *m/z* 428 $[M + H]^+$.

3′-[*cis*-4-(Boc-amino)cyclohexanecarboxylic Acid] Tetraacetylquercetin Ester (5d). Yield: 80%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.37 (s, 9H, CH_3), 1.42−1.84 (br m, 6H, CH_2), 1.88−2.10 (m, 2H, CH_2), 2.21 (s, 3H, 3-OAc), 2.32 (m, 9H, aromatic-OAc), 2.85 (br, 1H, CH), 3.41 (br, 1H, CH), 6.88 (br d, 1H, NH), 7.17 (d, 1H, J = 2.2 Hz), 7.53 (d, 1H, H-5′, J = 8.2 Hz), 7.65 (d, 1H, J = 2.2 Hz), 7.76−7.93 (m, 2H, H-2′, H-6′). MS (ESI-ion trap): *m/z* 718 $[M + Na]^+$, *m/z* 734 $[M + K]^+$, *m/z* 640 $[MH-CH_2{=}C(CH_3)_2]^+$.

3′-[*cis*-4-Amino-cyclohexanecarboxylic Acid] Tetraacetylquercetin Ester Hydrochloride (6d). Yield: 75%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.51−1.95 (br m, 6H, CH_2), 1.98−2.20 (br m, 2H, CH_2), 2.07 (s, 3H, 3-OAc), 2.32 (m, 9H, aromatic-OAc), 3.03 (br, 1H, CH), 4.26 (br, 1H, CH), 7.17 (d, 1H, J = 2.2 Hz), 7.55 (d, 1H, H-5′, J = 8.2 Hz), 7.64 (d, 1H, J = 2.2 Hz), 7.76−7.94 (m br, 5H, H-2′, H-6′, $^+NH_3$). MS (ESI-ion trap): *m/z* 596 $[M + H]^+$.

3′-[4-(Boc-amino)benzoic Acid] Quercetin Ester (3e). Eluents of flash chromatography: $CHCl_3/CH_3OH$ 9:1. Yield: 12%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.49 (s, 9H, CH_3), 6.18 (d, 1H, J = 1.65 Hz), 6.45 (d, 1H, J = 1.6 Hz), 7.11 (d, 1H, H-5′, J = 8.2 Hz), 7.58−7.75 (m, 2H), 7.91−8.12 (m, 4H), 9.88 (s, 1H, NH). MS (ESI-ion trap): *m/z* 522 $[M + H]^+$.

3′-[4-Aminobenzoic Acid] Quercetin Ester Hydrochloride (4e). Yield: 70%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 5.00 (br, $^+NH_3$), 6.19 (d, 1H, J = 2.2 Hz), 6.47 (d, 1H, J = 2.2 Hz), 6.63−6.81 (m, 2H), 7.11 (d, 1H, H-5′, J = 8.2 Hz), 7.76−7.88 (m, 2H), 7.91 (d, 1H, H-2′, J = 2.2 Hz), 7.97 (dd, 1H, H-6′, J = 8.2, 2.2 Hz). MS (ESI-ion trap): *m/z* 422 $[M + H]^+$.

3′-[4-(Boc-amino)benzoic Acid] Tetraacetylquercetin Ester (5e). Yield: 75%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 1.49 (s, 9H, CH_3), 2.18 (s, 3H, 3-OAc), 2.28−2.41 (m, 9H, aromatic-OAc), 7.16 (d, 1H, J = 2.2 Hz), 7.57 (d, 1H, H-5′, J = 8.2 Hz), 7.63−7.77 (m, 3H), 7.85−7.95 (m, 2H), 7.96−8.10 (m, 2H, H-2′, H-6′), 9.94 (s, 1H, NH). MS (ESI-ion trap): *m/z* 690 $[M + H]^+$, *m/z* 712 $[M + Na]^+$, *m/z* 728 $[M + K]^+$, *m/z* 634 $[MH-CH_2{=}C(CH_3)_2]^+$.

3′-[4-Aminobenzoic Acid] Tetraacetylquercetin Ester Hydrochloride (6e). Yield: 53%. ^{1}H NMR (250 MHz, DMSO-d_6) δ (ppm): 2.18 (s, 3H, 3-OAc), 2.31−2.38 (m, 9H, aromatic-OAc),

4.58 (br, $^{+}NH_3$), 6.60−6.74 (m, 2H), 7.16 (d, 1H, J = 2.2 Hz), 7.53 (d, 1H, H-5′, J = 8.8 Hz), 7.67 (d, 1H, J = 2.2 Hz), 7.72−7.82 (m, 2H), 7.85 (dd, 1H, H-6′, J = 8.8, 2.2 Hz), 7.93 (d, 1H, H-2′, J = 2.2 Hz). MS (ESI-ion trap): m/z 590 $[M + H]^+$, m/z 548 $[MH-CH_2{=}O]^+$.

4.2. Chemical Stability Studies. 4.2.1. UV−vis Spectrophotometry. The chemical stability of the synthesized compounds in water-containing solutions was tested following changes in the UV−vis spectra between 190 and 500 nm in PBS (20 mM, pH 7.0, μ = 0.1 M with KCl)/CH_3CN 1:1 mixtures at 25 °C. The reaction was initiated by adding 100 μL of a freshly prepared solution of the selected substrate in acetonitrile to 3 mL of a PBS/CH_3CN mixture to give a final concentration in the $(3-8) \times 10^{-5}$ M range. Spectral changes were followed with a Perkin-Elmer Lambda 5 spectrophotometer equipped with water-thermostated cell holders. Quartz cells with an optical path of 1 cm were used for all measurements. The observed rate constants for the hydrolysis of **3a**−**d** and **4a**−**d** were obtained by following the absorption changes at 255.2 nm for at least $3t_{1/2}$ and by interpolating the absorbance readings versus time with eq 1.

$$A = A_\infty + (A_0 - A_\infty)e^{-k_{obs}t} \quad (1)$$

In the case of two sequential processes, as observed for derivatives **6a**−**d**, the reactions were followed at 396.0 nm. Interpolation according with eq 2, where [Int] is the concentration of the intermediate product, tetraacetylquercetin, and ϵ_{Int} is its molar absorbtivity, provided the observed rate constants for the two consecutive hydrolysis steps, $k_{1,obs}$ and $k_{2,obs}$. All determinations were performed at least twice.

$$A = A_0e^{-k_{1,obs}t} + \frac{\epsilon_{Int}k_{1,obs}}{k_{2,obs} - k_{1,obs}}[Int](e^{-k_{1,obs}t} - e^{-k_{2,obs}t}) + A_\infty\left(\frac{k_{2,obs}(1 - e^{-k_{1,obs}t}) - k_{1,obs}(1 - e^{-k_{2,obs}t})}{k_{2,obs} - k_{1,obs}}\right) \quad (2)$$

4.2.2. HPLC−UV and LC−MS. The hydrolysis of **6a**−**d** was monitored by HPLC−UV and LC−ESI-MS to identify the products. The reaction, carried out as described in the preceding paragraph, was quenched at different times by adding 3 μL of pure HCOOH. Chromatography was also used to verify the stability of compounds **3e**, **4e**, **5e**, and **6e**. In the case of the first two, the absorption spectra are undistinguishable from those of a 1:1 mixture of quercetin and 4-(Boc-amino)benzoic acid, the theoretical hydrolysis products. HPLC−UV analyses were performed with the Thermo Separation Products system mentioned above. The sample solution (20 μL) was injected into a reversed-phase column (Synergi-MAX, 4 μm, 150 mm × 4.6 mm i.d.; Phenomenex). Solvents A and B were H_2O/CH_3CN 9:1 and CH_3CN both containing 0.2% HCOOH: the acid was necessary to avoid hydrolytic processes inside the column. The gradient for B was as follows: from 20% to 30% in 15 min and then to 100% in 15 min; the flow rate was 1 mL/min. Chromatograms were obtained plotting data recorded at 300 and 370 nm. LC−MS was performed on selected samples with a 1100 Series Agilent Technologies system, equipped with binary pump (G1312A), diode array detector (G1315B), and MSD SL Trap mass spectrometer (G2445D SL) with ESI source operating in full-scan mode from 100 to 1500 m/z in positive ion mode. The column, solvents, and gradient profile were the same as those used for HPLC−UV analyses.

4.3. Solubility in HBSS. About 1 mg of compounds **2**, **5a**−**e**, and **6e** was added to 20 mL of HBSS and sonicated for 10 min. The mixtures were then allowed to settle for 24 h, and the supernatants were analyzed by HPLC−UV.

4.4. Cell Assays. 4.4.1. Materials. All chemicals for buffer preparations were of laboratory grade, obtained from J. T. Baker, Merck, or Sigma. The composition of HBSS was as follows (in mM units): NaCl 136.9, KCl 5.36, $CaCl_2$ 1.26, $MgSO_4$ 0.81, KH_2PO_4 0.44, Na_2HPO_4 0.34, glucose 5.55, pH 7.4 (with NaOH). Corning−Costar Transwell Clear plates (12- or 6-well, polyester membrane, 12 or 24 mm insert diameter, 1.0 or 4.7 cm^2 septum surface, respectively, according to the producer; 0.4 μm pore size) were purchased from Cellbio.

MDCK-1, MDCK-2, and Caco-2 cell lines were kindly provided by the groups of Professors C. Montecucco, M. De Bernard, S. Garbisa, and E. Papini of the Department of Biomedical Sciences, University of Padova. Another Caco-2 clone, originally from ATCC, was purchased from the Istituto Zooprofilattico di Brescia (Italy). MDCK (Madin−Darby Canine Kidney) cells derive from dog kidney. MDCK-1 cells form tight, high-resistance (up to 20 kΩ·cm^2) epithelia. The closely related MDCK-2 line originated from the same tissue but has "leaky" tight junctions and produces monolayers with approximately 50-fold lower trans epithelial resistance (TER). The difference is due to the expression by MDCK-2 cells of zonula occludens protein claudin 2 in addition to claudins 1 and 4.[93] Caco-2 is a human colorectal adenocarcinoma line, known to exhibit heterogeneity. Its monolayers characteristically exhibit TER values of a few hundred Ω·cm^2.

4.4.2. Cell Culture and Monolayer Formation. Cells were seeded in culture flasks and passaged in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Biospa), 1% penicillin/streptomycin solution (10 000 U/mL and 10 mg/mL, respectively, in PBS), 1% glutamine (200 mM in PBS), 1% nonessential amino acids (100× solution), and 1% Hepes (1 M in PBS). The cells were seeded onto tissue culture-treated Transwell Clear inserts at a density of 3×10^5 cells per cm^{-2} for MDCK-1 and Caco-2 and 5×10^5 cells per cm^{-2} for MDCK-2. Monolayers were grown in a humidified atmosphere of 5% CO_2 at 37 °C. TER was measured periodically using a Millipore Millicell-ERS epithelial volt−ohmmeter, and experiments were performed when values reached approximately 10−15 kΩ·cm^2 for MDCK-1, 100−300 Ω·cm^2 for MDCK-2, and 1.5−2 kΩ·cm^2 for Caco-2 (10−20 days postseeding). These values are in line with literature data,[94−96] indicating the formation of epithelia with the appropriate "tightness" of cell−cell junctions. This property was also assessed measuring the diffusion of Lucifer yellow from the apical to the basolateral compartment[97] (see the Supporting Information).

4.4.3. Transport across Cell Monolayers. The cell layers were washed twice with warm HBSS. The initial stock solution of compounds **1**, **2**, **5a**−**e**, and **6e** was freshly made in DMSO and was then diluted with HBSS to give formally 30−50 μM solutions (final DMSO concentration 0.1%) and sonicated to obtain a finer dispersion of undissolved suspended material. This loading solution was added to the apical side (0.5 or 1.5 mL for 12-mm and 24-mm septa, respectively), while HBSS was added on the basolateral side (1.5 and 2.6 mL, respectively). In most instances the compound was added simultaneously to the apical compartments of four inserts, and the apical and basolateral solutions were collected after 0.5, 1.5, 3, and 6 h. In other cases samples were obtained only at 3 or 6 h.

Apical and basolateral solutions were transferred into glass vials and frozen for further analysis by HPLC−UV and LC−MS. In some cases the Transwell inserts (cells and supporting septum) were extracted with 1 mL of methanol containing ascorbic acid (1 mM) by sonication for 20 min. The solution was filtered through 0.45 μm PTFE syringe filters (Chemtek analytica) and stored at −20 °C until analyzed. Controls showed that filtration did not result in any loss of solutes.

Control experiments were run by incubating the cells in the presence of only DMSO at a final concentration of 0.1% in HBSS to assess background signals in the HPLC analyses. Additional controls were run by incubating each compound in the absence of cells to verify the chemical stability of the compounds under the conditions used for transport experiments.

Basolateral solutions were analyzed without any treatment; apical solutions were diluted with an equal volume of CH_3CN and filtered through 0.45 μm PTFE filters to eliminate cell residues. HPLC−UV and LC−MS analyses were carried out with the same instruments and the same column described above for the chemical stability studies but with different solvents and a different gradient. Solvent A was H_2O/THF/TFA (98:2:0.1, v/v/v) and B was CH_3-

CN. The flow rate was 1 mL/min; the gradient for B was as follows: 17% (2 min), 25% (in 5 min), 35% (in 8 min), 50% (in 5 min), 100% (in 15 min). The eluate was preferentially monitored at 270, 300, and 370 nm. Due to their spectral characteristics, quercetin and its sulfated derivatives were best observed at 370 nm, acylquercetins and quercetin-*o*-quinone at 300 nm. All compounds, except the quinone, were however visible, with lower sensitivity, also at the other wavelength. LC−MS was performed on selected samples both in positive and negative ion mode.

Metabolites were identified by their UV−vis spectra, mass spectra, the use of a 4′-methylquercetin standard, and experiments with sulfatase (in the case of sulfates). These last experiments consisted in the treatment of portions of selected samples with sulfatase (*Aerobacter aerogenes*, Sigma-Aldrich), followed by comparison of HPLC traces with those of untreated portions. A solution (400 μL) of the sample was concentrated to half-volume and incubated with sulfatase (0.4 U, 25 μL) at 37 °C for 30 min. After addition of 200 μL of methanol containing 1 mM ascorbic acid, the sample was centrifuged (13 600*g*, 4 °C, 5 min), filtered through 0.45 μm PTFE syringe filters, and analyzed by HPLC−UV.

For quantification purposes, the concentration was calculated on the basis of standard curves. The quantification limit on the diode array detector was determined to be 0.2 μM. The calibration curve built using **1** was also used for quercetin sulfate, methylquercetin sulfate, and acetylquercetin, assuming for these compounds the same absorption coefficient ϵ_{370} of quercetin; the calibration curve of **2** was used for diacetylquercetin assuming ϵ_{300} to be similar to that of pentaacetylquercetin. 3′-Boc-amino acid triacetyl quercetin esters were quantified using the calibration curve of the corresponding 3′-Boc-amino acid tetraacetyl quercetin esters, assuming the same absorption coefficient at 300 nm.

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Supporting Information Available: Elemental analyses of the compounds and tests of monolayer permeability to Lucifer yellow. This material is available free of charge via the Internet at http://pubs.acs.org.

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