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# Remarkable Stability and Cytostatic Effect of a Quercetin Conjugate, 3,7-Bis-*O*-Pivaloxymethyl (POM) Quercetin

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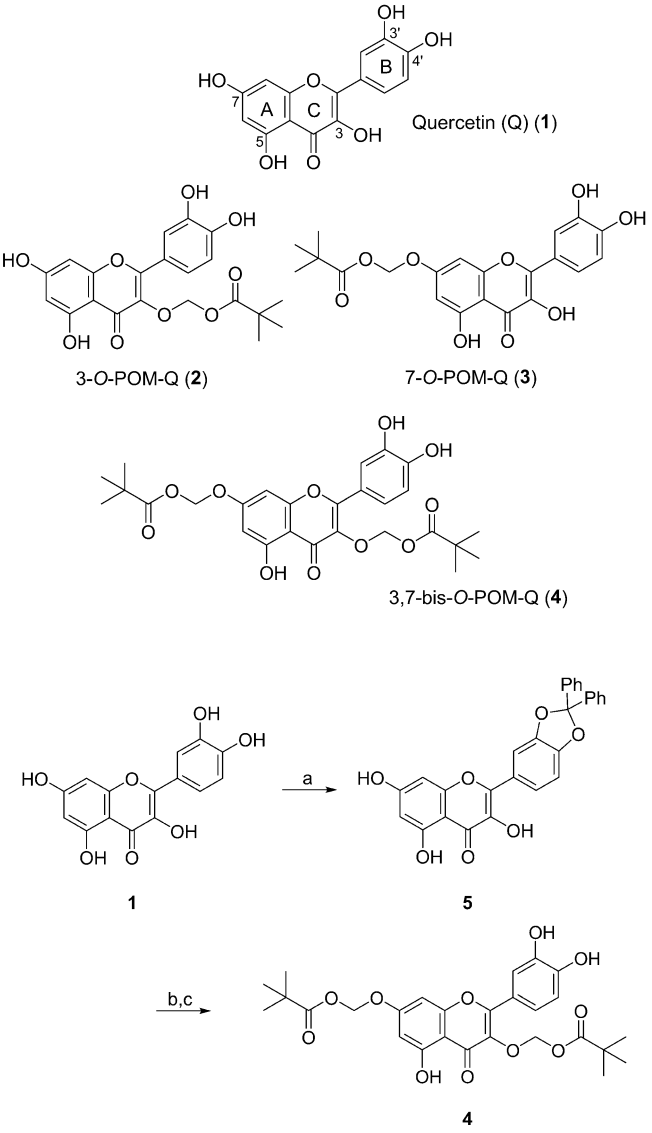
Quercetin (Q, **1**) is a polyphenolic flavonoid that is readily found in human diet. The tremendous growth in the study of this bioactive compound has revealed numerous health-promoting effects, such as antioxidant,[1] antiviral,[2,3] and anticancer[4] activities. However, quercetin is unstable and undergoes oxidative decomposition in aqueous solution.[5–7] As a result, the bioactivity of quercetin is often limited by its stability, and it is desirable to develop a method to increase quercetin stability.

Many studies support the critical role of the free hydroxy group in the 3 position in the oxidative decomposition of quercetin.[8,9] In our previous study,[10] we introduced a pivaloxymethyl (POM)[11,12] group to quercetin at this position and observed remarkable stability of the resulting 3-*O*-POM-Q (**2**). Quercetin–POM conjugate **2** showed very slow decomposition ($t_{1/2}=52$ h) in a cell culture medium under conditions of high oxidative stress. This remarkably stable quercetin–POM conjugate, however, failed to show cytostatic effects against various cancer cell lines due to its inability to permeate the cell membrane. Conversely, 7-*O*-POM-Q (**3**) with the POM group attached to the hydroxy group in the 7 position of quercetin showed much faster hydrolysis and/or decomposition in cell culture medium ($t_{1/2}=4$ h) compared with 3-*O*-POM-Q (**2**). However, unlike 3-*O*-POM-Q (**2**), 7-*O*-POM-Q (**3**) showed efficient cellular uptake and intracellular conversion to quercetin and its metabolites, which resulted in enhanced cytostatic effects against cancer cell lines compared with the parent compound, quercetin.

We reasoned that the introduction of POM groups at both the 3- and 7-hydroxys of quercetin would produce synergistic effects to provide a stable quercetin–POM conjugate with efficient cellular uptake and thereby significant cytostatic activity. Herein, we report the synthesis and biological evaluation of 3,7-bis-*O*-POM-Q (**4**).

The synthesis of **4** was accomplished by nucleophilic substitution of the selectively protected quercetin, quercetin diphenylmethylketal (**5**), with excess amount of pivaloxymethyl iodide (POM-I) (Scheme 1).

The stability of the quercetin–POM conjugates **2**–**4** was assessed in phosphate buffered saline (PBS; pH 7.4) and Dulbecco's modified eagle medium supplemented with fetal bovine serum (cDMEM), and the results are summarized in Table 1 and Figure 1.




**Scheme 1.** Synthesis of 3,7-bis-*O*-POM-Q (**4**). *Reagents and conditions*: a) $Ph_2CCl_2$, 180 °C, 30 min, 35%; b) POM-I, $K_2CO_3$, acetone, RT, 4 h; c) $H_2$, Pd/C, THF/MeOH (1:1), RT, 12 h, 63%.

The instability of quercetin in aerobic aqueous media such as PBS ($t_{1/2}=10$ h, Table 1) and cDMEM ($t_{1/2}<30$ min, Table 1) is well known.[10,13,14] In contrast, 3,7-bis-*O*-POM-Q (**4**) is as stable as the previously reported quercetin–POM conjugates **2** and **3** (Table 1)[10] against hydrolytic cleavage or oxidative decomposition in PBS. More intriguingly, in cDMEM, 3,7-bis-*O*-POM-Q (**4**) was even more stable ($t_{1/2}=100$ h, Table 1) than conjugates **2** ($t_{1/2}=52$ h, Table 1) and **3** ($t_{1/2}=4$ h, Table 1).[10] High-performance liquid chromatography (HPLC) analysis of the cell cul-

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**Table 1.** Stability of quercetin and quercetin–POM conjugates in different media.[a]

| Compd | Half-life ($t_{1/2}$) [h] PBS | cDMEM |
|---|---|---|
| Q (**1**)[b] | 10 | < 30 min |
| 3-*O*-POM-Q (**2**)[b] | > 24 | 52 |
| 7-*O*-POM-Q (**3**)[b] | > 24 | 4 |
| 3,7-bis-*O*-POM-Q (**4**) | > 24 | 100[c] |

[a] Phosphate-buffered saline (PBS); Dulbecco's modified eagle medium supplemented with fetal bovine serum (cDMEM). [b] Data taken from Reference [10]. [c] Conversion to 3-*O*-POM-Q (**2**) not to quercetin.

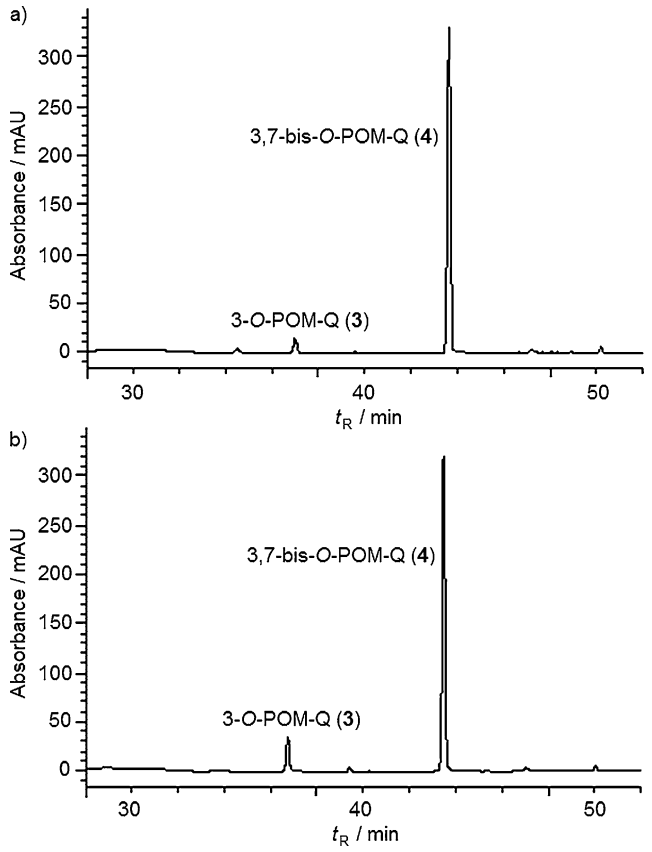


**Figure 1.** HPLC–UV chromatograms (340 nm) of 3,7-bis-*O*-POM-Q (**4**) after incubation in cell-free cDMEM for a period of a) 1 h and b) 24 h.

ture media after 24 h (Figure 1 b) showed less than 10% of 3,7-bis-*O*-POM-Q (**4**) was converted into its hydrolysis product, 3-*O*-POM-Q (**2**), but neither 7-*O*-POM-Q (**3**) nor quercetin was observed in the HPLC chromatogram (Figure 1). The exceptional stability of **4** in comparison with the mono-POM–quercetin derivatives **2** and **3** suggests the synergistic effect of the POM groups attached at the 3-hydroxy as well as the 7-hydroxy group. Presumably, the two POM groups do not allow 3,7-bis-*O*-POM-Q (**4**) to serve as a substrate for hydrolyzing enzymes present in cDMEM.

With the proven stability of **4** in cell culture media in hand, we then evaluated its cellular uptake. Quercetin itself is not fluorescent but, upon binding to cellular target proteins, it exhibits specific fluorescence ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500–540 nm).[15–18] In our previous study,[10] we showed that detection of fluorescence inside the cell can be used as an efficient method to evaluate the cellular uptake of quercetin and its conjugates. Thus, after incubation of cells with **4**, fluorescent staining was observed in a confocal microscope at two different time points (1 h and 12 h; Figure 2).

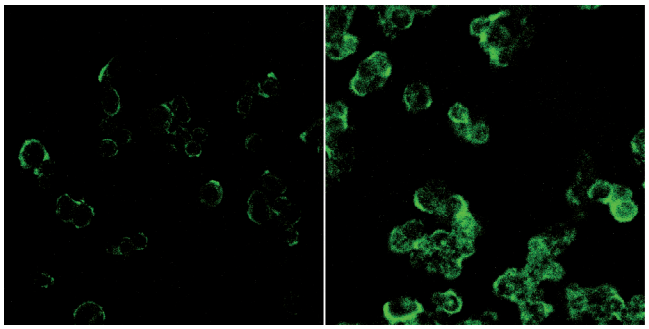

**Figure 2.** Confocal microscope images of fluorescent staining ($\lambda_{ex}$ = 488 nm, $\lambda_{em}$ = 500–540 nm) in HCT116 cells treated with 3,7-bis-*O*-POM-Q (**4**) in phosphate-buffered saline (3 μM) for 1 h (left panel) and 12 h (right panel).

Due to the hydrophobic nature of the POM moiety, 3,7-bis-*O*-POM (**4**) is largely membrane-associated, which can be observed by the fluorescence signal around the cell membrane (Figure 2). However, intensified cytoplasmic fluorescence signal observed 12 h after incubation (right panel, Figure 2) clearly demonstrates the time-dependent cellular uptake as well as intracellular localization of the quercetin derivatives.

The intracellular localization of 3,7-bis-*O*-POM-Q (**4**) and its hydrolysis product 3-*O*-POM-Q (**2**) was also confirmed by HPLC analysis of the cell lysate (Figure 3).

Knowing that 3-*O*-POM-Q (**2**) is not transported into the cell,[10] it is clear that 3-*O*-POM-Q (**2**) found in the cell lysate is a result of hydrolysis of intracellularly localized **4**; no trace of 7-*O*-POM-Q (**3**) or quercetin (i.e., metabolites) was observed. The favorable properties associated with 3-*O*-POM-Q (**2**) and 7-*O*-POM-Q (**3**), i.e., high stability and efficient cellular uptake, respectively, are combined in 3,7-bis-*O*-POM-Q (**4**). However, unlike 7-*O*-POM-Q (**3**), which was hydrolyzed to bioactive quercetin,[10] 3,7-bis-*O*-POM-Q (**4**) did not convert to quercetin inside the cell.

Based on these results, we can conclude that the cellular uptake of the POM-substituted quercetin derivatives depends on the specific location of the POM group at the 7-hydroxy position. Therefore, rather than nonspecific passive transport, a transporter-mediated active transport is the more plausible mechanism involved in the selective cellular uptake of the quercetin conjugates with POM groups at the 7-hydroxy position.

The cytostatic effect of 3,7-bis-*O*-POM-Q (**4**) was next evaluated. Cell lines derived from various types of cancer, including breast (MCF-7), colon (HCT116), and prostate cancer (DU145), as well as normal human diploid fibroblast cells (HS27) were treated with quercetin and quercetin–POM conjugate **4**, and

  

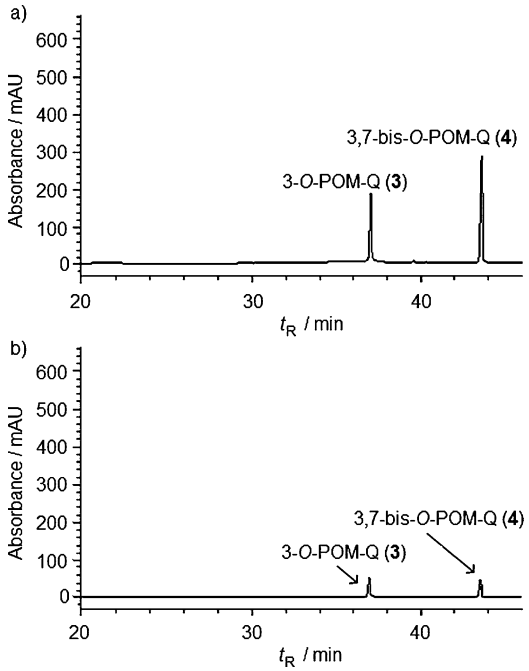


**Figure 3.** HPLC chromatograms of a) cell culture medium and b) cell lysate after 12 h of incubation of MCF-7 cells with the quercetin conjugate 3,7-bis-*O*-POM-Q (**4**).

the cell viabilities were estimated using an MTT assay (Figure 4).

In order to demonstrate the stability of quercetin–POM conjugate **4** against extracellular oxidative stress, no antioxidant additive was used. Under these assay conditions, quercetin did not inhibit cell proliferation, whereas the **4** showed significant cytostatic activity in all three cancer cell lines tested (Figure 4a–c). The lack of cytostatic effect by quercetin is not surprising because it is known to be inactive in cell culture media without stabilizing agent such as ascorbic acid.[5–7] However, under the same conditions, the cells treated with 3,7-bis-*O*-POM-Q (**4**) showed significantly reduced viability. The cytostatic effect of **4** was specific to cancer cells, and the viability of the normal human diploid fibroblast cell line (HS27) was unaffected by **4** (Figure 4d).

To compare the inhibitory mechanism of quercetin and 3,7-bis-*O*-POM-Q (**4**) on cell proliferation, the effects of these compounds on the cell cycle were investigated (Figure 5). Quercetin is known to arrest the cell cycle in the S/early G2 phase.[19] As reported, 12 h after addition of quercetin, the percentage of cells in the S phase increased with concurrent decrease of cells in the G0/G1 as well as the G2/M phases (Figure 5b, d), meaning that the S to G2/M progression was arrested. The seemingly unrealistic cell cycle arrest by the unstable quercetin ($t_{1/2} < 30$ min in cell culture medium, Table 1) 12 h after incubation can be understood in the context of efficient cellular uptake of quercetin followed by its intracellular metabolism. Previously,[10] we observed quercetin and its metabolites (glucuronides, sulfates, and methyl derivatives) remaining in the cytoplasm up to 12 h after incubation, and the sustained efficacious level of quercetin and/or its metabolites inside the cell must cause cell cycle arrest. In contrast, cells treated with quercetin–POM conjugate **4** showed low populations in the S phase but an increased percentage of cells in the G0/G1 phase (Figure 5c, d), which can be attributed to cell cycle arrest in the G0/G1 phase.

In conclusion, we have shown that 3,7-bis-*O*-POM-Q (**4**) exhibits remarkable stability in cell culture medium and efficient cellular uptake. Inside the cell, it is selectively converted into the mono-hydrolysis product, 3-*O*-POM-Q (**2**), but is not hydrolyzed further to give the bioactive parent compound, quercetin. However, intracellularly localized **4** and/or **2** exhibit significant inhibition of cell proliferation via a different mechanism compared with that of quercetin. Taken together, this result implies that stable quercetin–POM conjugate **4** has potential to serve as an anticancer agent.

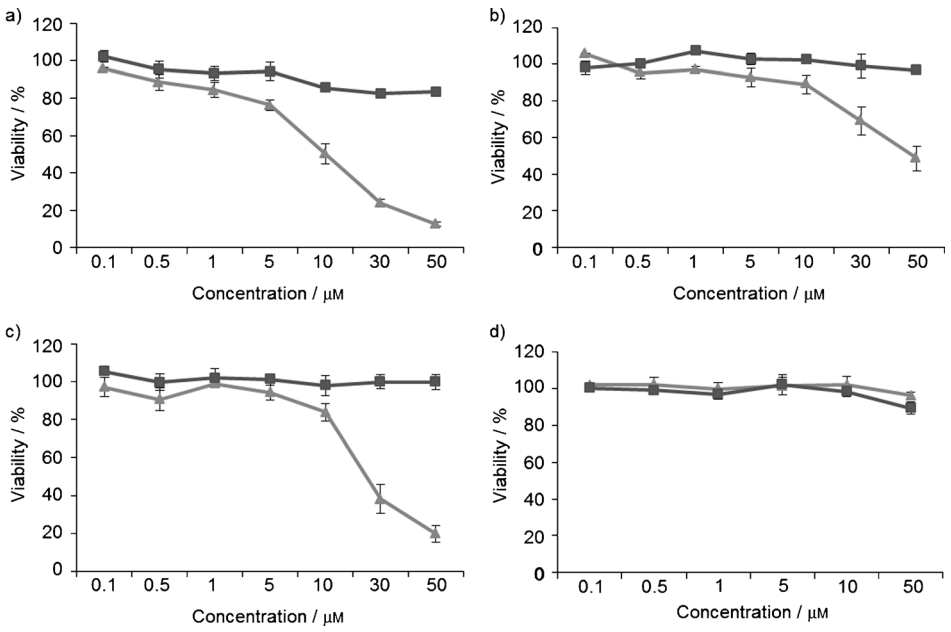


**Figure 4.** Viability of a) MCF-7, b) HCT116, c) DU 145, and d) HS27 cells treated with quercetin (**1**) [■] and 3,7-bis-*O*-POM-Q (**4**) [▲].

## Experimental Section

Full details of the synthesis of intermediate **5** and 3,7-bis-*O*-POM-Q (**4**) are given in the Supporting Information along with protocols for the stability and biological assays used in this study.

  

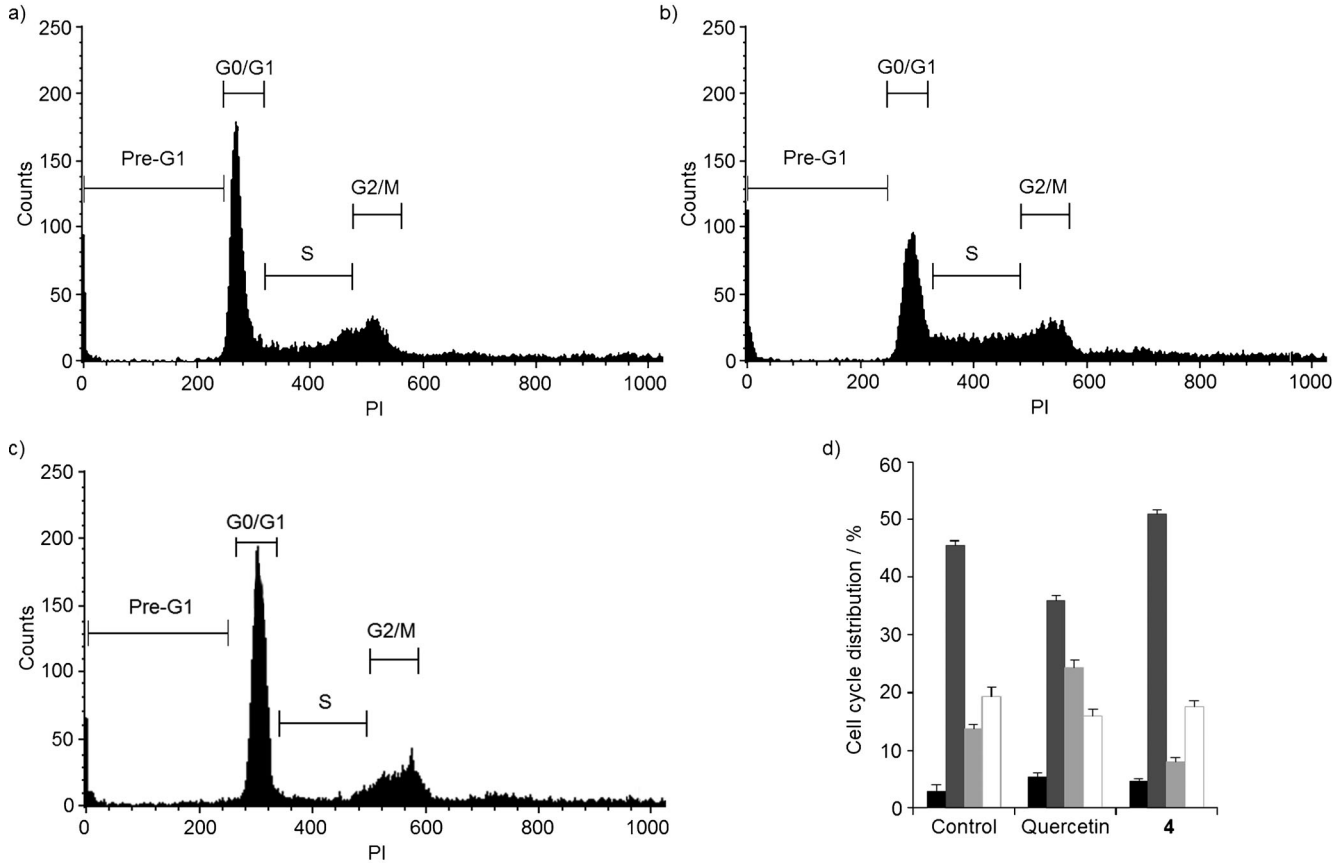


**Figure 5.** Cell cycle analysis of MCF-7 cells by propidium iodide (PI) staining and flow cytometry after treatment with a) 1% DMSO, b) 100 μM of quercetin, c) 30 μM of 3,7-bis-*O*-POM-Q (**4**) for 12 h. d) The percentage of cells in each phase of the cell cycle: pre-G1 (■), G0/G1 (■), S (■), G2/M (□).

## *Acknowledgements*

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