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Inhibition of Protein Phosphatases by Michael Adducts of Ascorbic Acid Analogues with α,β-Unsaturated Carbonyl Compounds†

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Abstract—By investigating the stereospecific Michael reaction of derivatives of ascorbic acid with acrolein we obtained a novel class of protein Ser/Thr phosphatase inhibitors. The inhibitory effect of the Michael adducts was examined using the canonical protein phosphatases type 1, 2A and 2B. Of the isozymes examined the type 1 isoform was strongly inhibited.

Introduction

The reversible phosphorylation of serine, threonine and tyrosine containing proteins by phosphatases is a key mechanism for signal transduction.[1,2] This molecular 'on and off switch' regulates an extremely wide variety of cellular functions including cell division, hormone action, cell growth, glycogen metabolism and muscle contraction. These functions are regulated by a dynamic relationship between multiple protein kinase isozymes (currently more than 1000 isoforms) and a highly divergent group of protein phosphatases.[3–6] Considering the tumor promotion effects of phorbol esters which are mediated by protein kinase C, most synthetic efforts in this general area of cell regulation centered on protein kinases to possibly intervene at early stages of malignant transformations.

Initially, protein phosphatases were classified into Ser/Thr protein phosphatases, Tyr protein phosphatases and phosphatases with dual-specificity against Ser/Thr and Tyr residues. Ser/Thr protein phosphatases were subclassified as type 1 and type 2 enzymes[9] according to their substrate specificity, divalent metal-ion dependence and sensitivity toward specific protein inhibitors such as inhibitor-1,[7] inhibitor-2,[8] DARPP-32 and NIPP1; Table 1). Although the number of Ser/Thr protein phosphatases has vastly increased and exceeds the limits of this early classification, it nevertheless remains a useful scheme.

Our current knowledge about specific inhibitors of protein phosphatase isoforms is rudimentary. So far, three structurally different types of natural compounds have been found to inhibit: (1) terpenoids such as cantharidine and thyrsiferyl-23-acetate (Fig. 1), (2) cyclic peptides such as microcystin[10] and nodularin (Fig. 2) and (3) polyketides such as okadaic acid,[11] calyculin A[12] and tautomycin[13] (Fig. 3). Each of these substances inhibits at least two of the major subclass Ser/Thr protein phosphatases. Microcystin, okadaic acid, tautomycin and calyculin A mainly inhibit PP1 and PP2A isoforms. The IC_{50} values range between 0.1 and 50 nM.[14] The terpenoides cantharidine and thyrsiferyl-23-acetate were shown to be somewhat selective inhibitors of PP2A with IC_{50} values of 0.16–10 μM.

High toxicity, especially hepatotoxicity, commonly found with these natural Ser/Thr protein phosphatase inhibitors significantly limits their pharmacological use. In vivo, they also appear to be associated with a multitude of non-specific side effects. Furthermore, the supply of these natural compounds is rather restricted and despite some recent total synthesis efforts, the natural Ser/Thr phosphatase inhibitors are not available in quantities sufficient for large scale screening purposes.

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†Dedicated to Professor Dr. H. J. Roth, Tübingen, Germany, on the occasion of his 70th birthday.

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Table 1. Ser/Thr protein phosphatase classification

Sub-family	Specificity for phosphorylase kinase	Metal-ion dependence	Inhibition by inhibitor-1 and -2
PP1	β-subunit	No	Yes
PP2A	α-subunit	No	No
PP2B	α-subunit	Yes, Ca^{2+}	No
PP2C	α-subunit	Yes, Mg^{2+}	No

Since we know phosphatases are active participants in essentially all signal transduction pathways, the development of selective, non-toxic and cell-permeable inhibitors is very important to investigate their specific contribution. Such efforts may also lead to useful therapeutic agents in the future.

Synthetic Strategy

In 1983, Fodor et al. reported the ability of ascorbic acid to serve as a Michael donor in conjunction with a variety of α,β-unsaturated carbonyl compounds.[15] The reaction with acrolein stereospecifically leads to a tricyclic hemiacetal lactone **2**. Subsequently, Eger et al. studied the solvent dependent equilibrium between the spiro compound **1** and the pyranose **2** proceeding via intermediate **I** (Fig. 4).[16,17]

If the Michael addition of ascorbic acid to α,β-unsaturated carbonyl compounds is carried out with ascorbic acid derivatives with a blocked glycol function, the bicyclic spiro adducts should be selectively obtained as the formation of the respective pyranosid **2** is excluded. The spiro compounds possess a spiroacetal moiety which is comparable to those present in the protein phosphatase inhibitors okadaic acid, tautomycin and calyculin. Even though the structural similarity between these natural protein phosphatase inhibitors and the Michael adducts of ascorbic acid cannot be extended any further, we reasoned that we might generate enzyme inhibitors lacking of the toxic activities intrinsic to these natural compounds. Preliminary studies of the reaction of 6-*O*-palmitoyl-ascorbic acid with acrolein reported by Schmidt[18] gave the bicyclic furanosid spiro adduct **3**. However, in aqueous media the spiro compound rearranged upon addition of water to the respective pyranosid **4** (Fig. 5). In order to exclusively form the spiro products we wanted to further investigate this synthetic pathway by variation of the reaction conditions.

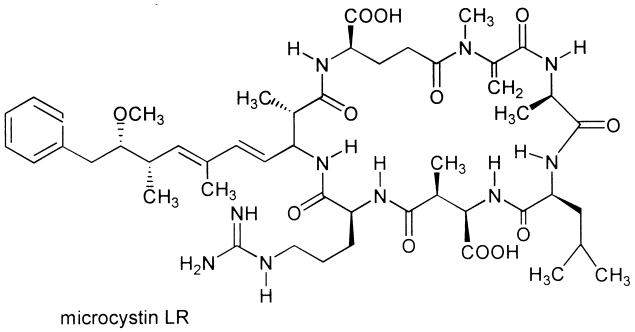


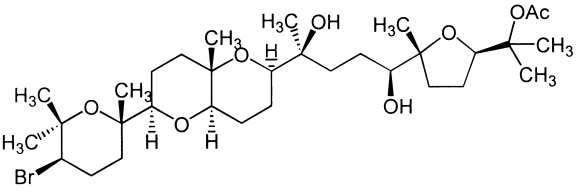
Figure 2. Natural cyclic peptide inhibitor of Ser/Thr protein phosphatases.

Results and Discussion

Initial attempts to react the Michael donor 6-*O*-palmitoyl-ascorbic acid with acrolein in absolute ethanol resulted upon recrystallisation from ethanol in a single product. This was not the expected spiro compound **3**, but the pyranosid **5** as evident from its ^{13}C NMR, which exhibited the characteristic ethoxy signals at 63.2 and 14.9 ppm. This result led us to take a closer look at the possible reaction mechanisms. The initial Michael addition of 6-*O*-palmitoyl-ascorbic acid to acrolein leads to intermediate **I**. The latter can undergo intramolecular cyclisation to the respective spiro compound **3**. In solution, this intramolecular semi acetal formation is fast and reversible. In the presence of a nucleophilic solvent, in this case ethanol, intermediate **I** is attacked by the primary alcohol at C3 forming a semi acetal, followed by intramolecular cyclisation to the stable pyranosid **5**. A corresponding reaction was observed with other primary alcohols such as methanol or *n*-propanol. In both instances the respective bicyclic pyranosids **6** and **7** were obtained as sole products after recrystallisation (Fig. 6).

If the reaction is carried out in *tert*-butanol, a non nucleophilic alcohol, the bicyclic spiro adduct **3** can be isolated. Treatment of the latter with methanol, ethanol or *n*-propanol as primary alcohols again yielded the pyranosid structures **6**, **5** or **7**, respectively (Fig. 7).

The use of the secondary alcohols isopropanol or cyclohexanol as solvents in the Michael-addition led to the mixed acetals **8** and **9**, with a spiro structure (Fig. 8). This was evident from the ^{13}C NMR spectrum which displayed only a single carbonyl signal at 206 ppm and none of the bands characteristic for the pyranosid compound.



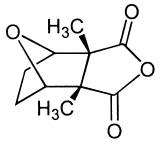


Figure 1. Natural terpenoid inhibitors of Ser/Thr protein phosphatases.

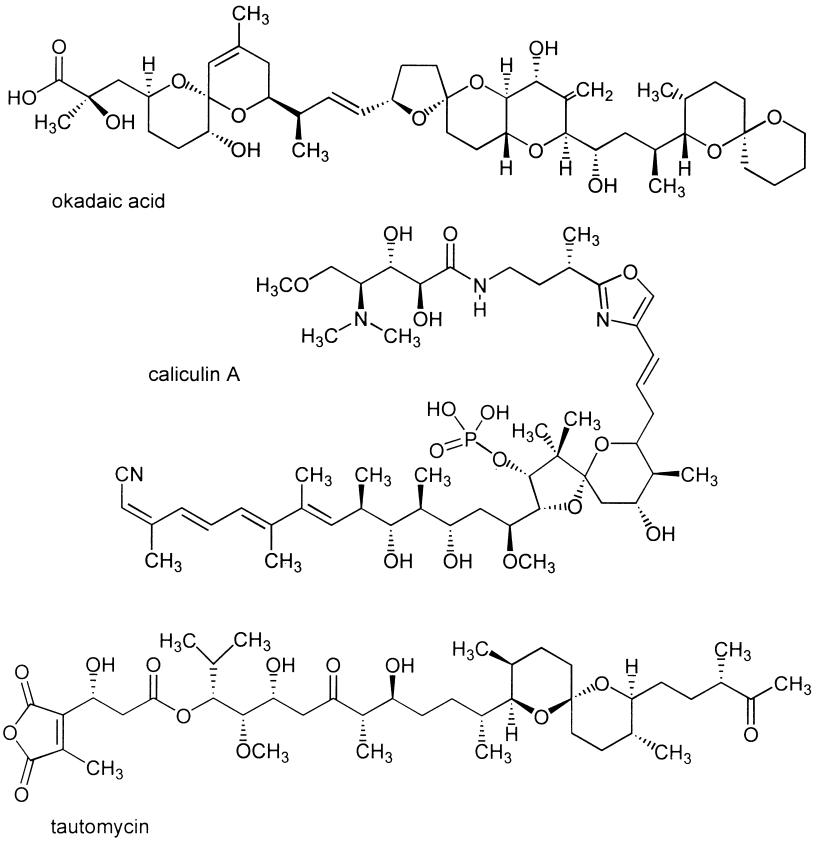


Figure 3. Natural polyketide inhibitors of Ser/Thr protein phosphatases.

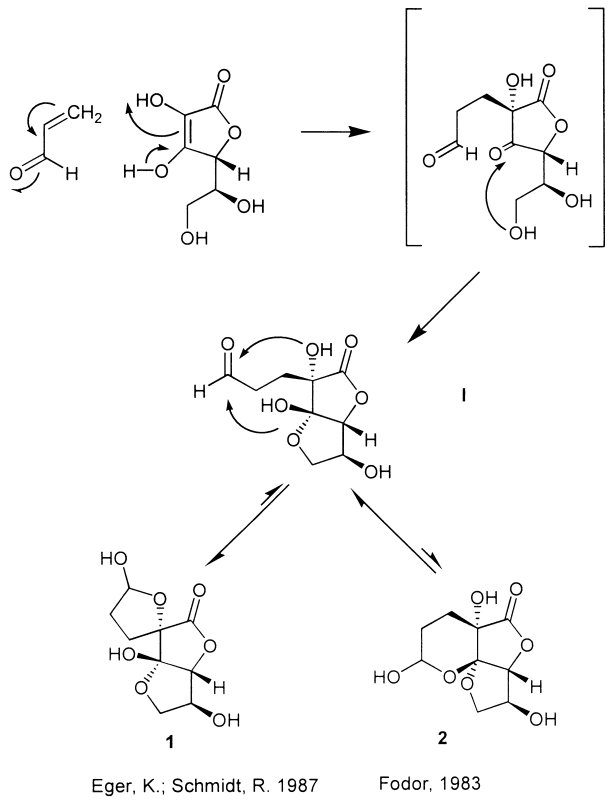


Figure 4.

The Michael addition of 5,6-*O*-dipalmitoyl-ascobic acid and acrolein in ethanol yielded a mixture of pyranosid and furanosid compounds, **10** and **11**, as determined by ^{13}C NMR.

We were unable to separate the product mixture. When *tert*-butanol was employed as an inert solvent, the reaction proceeded in analogy to that depicted in Figure 7 and only the furanosid spiro compound **10** was obtained (Fig. 9).

Finally, we investigated the ability of our Michael adducts to inhibit the catalytic activity of Ser/Thr phosphatases. At concentrations of up to 20 mM acid, alkaline and tyrosine phosphatase activities were unaffected. However, preliminary experiments demonstrated that a recombinant bovine protein phosphatase type 1 was inhibited by several Michael adducts of acrolein with ascorbic acid derivatives.[21] The extent of the inhibition was dependent on the lipophilicity of the generated compounds.[19,21] The positive results with compounds synthesized in this study are shown in Table 2. Further examination demonstrated that at these concentrations our adducts did not inhibit the catalytic activity of PP2A and PP2B (data not shown). The IC_{50} values for PP1 inhibition are in the low micromolar range, i.e. are about 10^3-fold higher than those reported for the natural compounds okadaic acid, tautomycin and calyculin.[10–13] Nevertheless, to our knowledge these are the first compounds which are

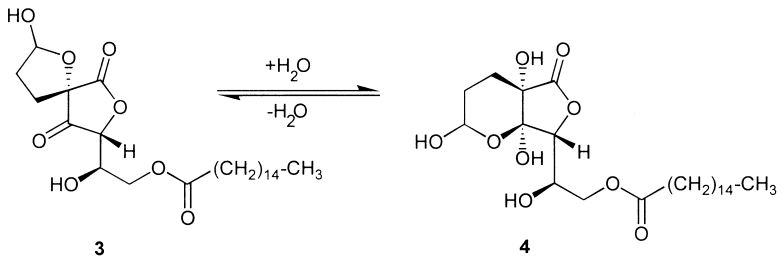


Figure 5.

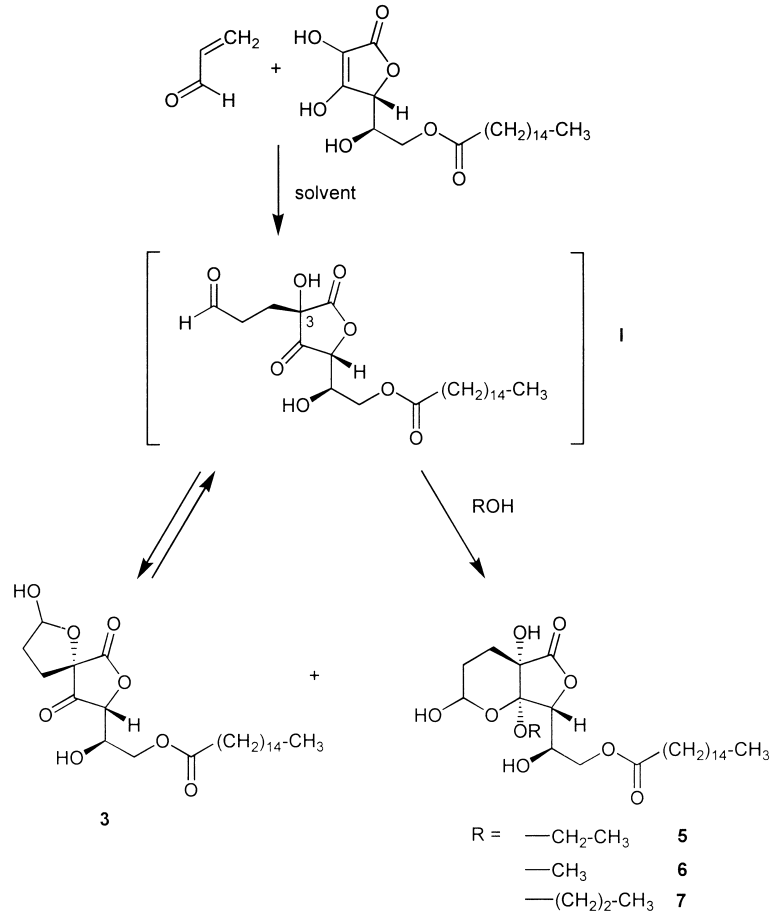


Figure 6.

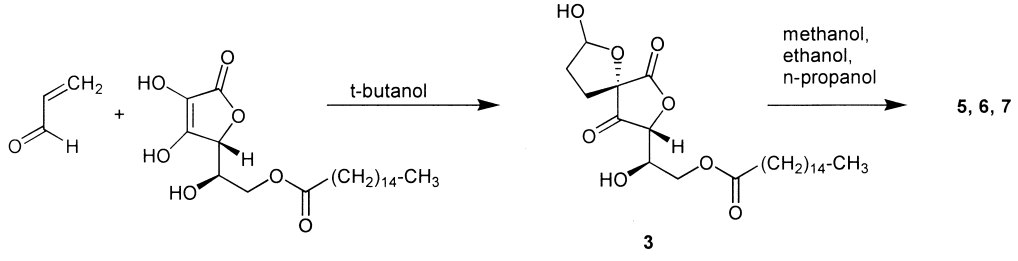


Figure 7.

easily accessible by synthetic means which inhibit protein Ser/Thr-protein phosphatases with a certain degree of specificity. Thus, we obtained a group of relatively non-toxic substances which inhibited PP1 at acceptable concentrations and with at least some specificity as far as other phosphatases are concerned. These results justify further efforts to modify and improve the inhibitor properties of this class of Michael adducts.

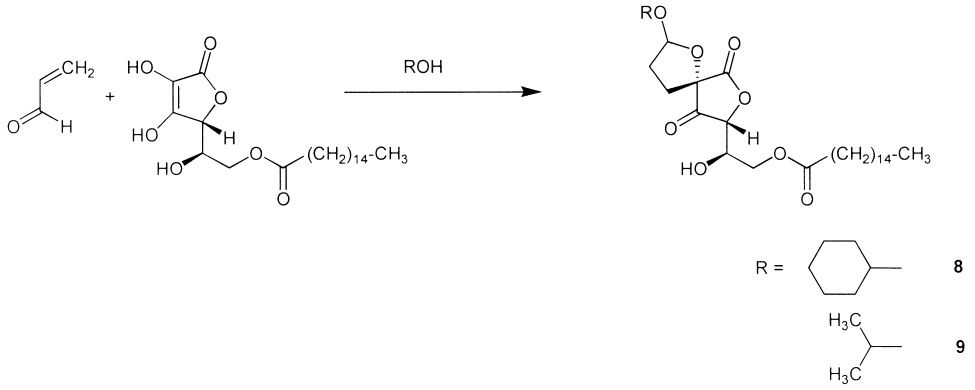


Figure 8.

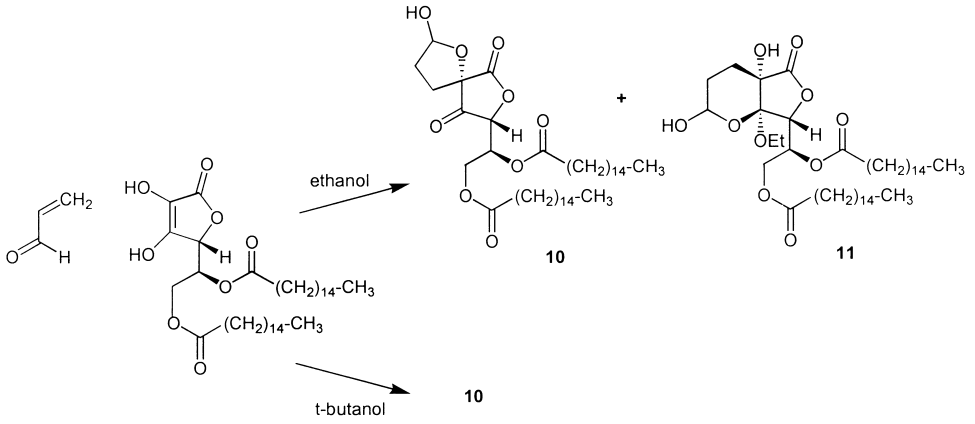


Figure 9.

Table 2. Inhibition of protein phosphatase type 1 activity by compound **5**, **6**, **9** and **10**/**11** compound[a]

Compound	IC_{50} (μM)
5	16
6	11
9	3.2
10/11	5.6

[a]Assays were carried out according to Cohen et al.[21,22]

Experimental

General methods

Melting points were determined on a Büchi 535 melting point apparatus, and are uncorrected. The IR spectra (KBr) were recorded on a Perkin–Elmer PC 16 FT-IR-spectrophotometer. The ^1H and ^{13}C NMR spectra were measured on a Varian Gemini 300 spectrometer (^1H: 300 MHz, ^{13}C: 75 MHz) using tetramethylsilan as an internal standard. Mass spectra were obtained by a Hewlett-Packard LC/MS-spectrometer (LC: HP 1050, MS: HP-MS-Engine 5989 A), a MALDI TOF (Perseptive Biosystems; Voyager-DERP) and by the Fast Atom Bombardement-methode (FAB) on a ZAB-HSQ VG Analytical Manchester spectrometer. Elementary analysis was carried out by a departmental unit.

(5′*S*, 8′*R*, 2*S*)-Hexadecane acid-2-hydroxy-2-(-2′-hydroxy-6′,9′-dioxo-1′,7′-dioxaspiro[4.4]non-8′-yl)-ethyl ester (3). Acrolein (1 mL, 15 mmol) was added dropwise under a nitrogen atmosphere to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry *tert*-butanol (40 mL). After stirring for 2 days at 70 °C the mixture was concentrated in vacuo to give 3.52 g (75%) of a colourless residue: IR (KBr) 3450, 2956, 2922, 1768, 1718 cm^{-1}; ^1H NMR: δ 5.88–5.77 (m, 2H); 5.17–5.07 (m, 2H); 4.48–4.14 (m, 3H); 2.43–1.32 (m, 32H); 0.93–0.89 (t, 3H, ^3J_H, H = 6.1Hz); ^{13}C NMR: δ 207.88, 173.83, 173.52, 102.55, 83.73, 80.19, 68.57, 64.18, 35.02, 34.36, 33.73, 32.57, 30.58, 30.16, 29.99, 29.81, 29.56, 29.04, 25.47, 23.26, 14.30; MS (Fab) *m*/*z* 472 $[M+1]^+$. Anal. calcd for $C_{25}H_{42}O_8$: C 63.81; H 9.00. Found C 63.58; H 8.88.

(4′a*S*, 7′*R*, 7′a*R*, 2*S*)-Hexadecane acid-2-hydroxy-2-(hexahydro-2′,4′a-dihydroxy-7′a-ethoxy-5′-oxo-2′H-furo[3,4-b]-pyran-7′-yl)-ethyl ester (5). Acrolein (1 mL, 15 mmol) was added dropwise under a nitrogen atmosphere to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry ethanol (40 mL). After stirring for 2 days at 70 °C the mixture was concentrated in vacuo and yielded a colourless residue. The residue was recrystallised from 10 mL of ethanol. A colourless powder, yield 2.72 g (53%), was obtained: mp 89 °C; IR (KBr) 3476, 3438, 3264, 2922, 2852, 1782, 1714 cm^{-1}; ^1H NMR: δ 7.32, 6.60, 6.55 (3s, 3H); 4.81 (m, 1H); 4.25–3.71 (m,

3H); 2.33–1.07 (m, 37H); 0.85 (t, 3H, $^3J_{H,H}=6.9$ Hz); ^{13}C NMR: δ 174.98, 172.68, 99.24, 95.54, 84.06, 69.91, 65.10, 64.05, 63.20, 33.35, 31.29, 29.00, 28.85, 28.67, 28.43, 27.36, 25.42, 24.33, 22.06, 14.93, 13.88; MS (EI) m/z 472 $[M\text{-OEt}]^+$. Anal. calcd for $C_{27}H_{48}O_9$: C 62.77; H 9.36; O 27.87; found C 62.37; H 9.53; O 28.10.

(4′a*S*, 7′*R*, 7′a*R*, 2*S*)-Hexadecane acid-2-hydroxy-2-(hexahydro-2′,4′a-dihydroxy-7′a-methoxy-5′-oxo-2′H-furo[3,4-b]pyran-7′-yl)-ethyl ester (6). Acrolein (1 mL, 15 mmol) was added dropwise under a nitrogen atmosphere to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry methanol (40 mL). After the mixture was stirred for 2 days at 70 °C it was concentrated in vacuo to give of a colourless residue which was recrystallised from 10 mL of methanol. A colourless powder, yield 2.2 g (44%), was obtained: mp 96 °C; IR (KBr) 3472, 3436, 2920, 2859, 1784, 1712 cm^{-1}; ^{1}H NMR: δ 4.70 (m, 1H); 4.26–4.04 (m, 5H); 3.32 (s, 3H); 2.34–1.07 (m, 32H); 0.85 (t, 3H, $^3J_{H,H}=6.1$ Hz); ^{13}C NMR: δ 174.90, 172.62, 99.31, 97.08, 84.03, 69.93, 65.14, 64.11, 56.15, 34.10, 31.95, 29.72, 29.68, 29.64, 29.49, 29.38, 29.28, 29.15, 24.82, 22.71, 14.13; MS (Fab) m/z 502 $[M]^+$; anal. calcd for $C_{26}H_{46}O_9$: C 62.13; H 9.22; O 28.65; found: C 62.15; H 9.11; O 28.80.

(4′a*S*, 7′*R*, 7′a*R*, 2*S*)-Hexadecane acid-2-hydroxy-2-(hexahydro-2′,4′a-dihydroxy-7′a-propyloxy-5′-oxo-2′H-furo-[3,4-b]pyran-7′-yl)-ethyl ester (7). Acrolein (1 mL, 15 mmol) was added dropwise under a nitrogen atmosphere to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry *n*-propanol (40 mL). After stirring for 2 days at 70 °C the mixture was concentrated in vacuo to give of a colourless residue. The residue was recrystallised from 10 mL of *n*-propanol. A colourless powder, yield 3.18 g (53%), was obtained: mp 90 °C; IR (KBr) 3434, 2958, 2922, 2852, 1784, 1712 cm^{-1}; ^{1}H NMR: δ 6.78, 6.58, 6.54 (3s, 3H); 4.81 (m, 1H); 4.25–3.71 (m, 5H); 2.33–1.07 (m, 37H); 0.85 (t, 3H, $^3J_{H,H}=$ 6.9 Hz); ^{13}C NMR: δ 174.96, 172.64, 99.25, 95.80, 84.06, 69.91, 69.46, 65.10, 64.06, 33.38, 31.28, 29.02, 28.98, 28.87, 28.69, 28.45, 24.34, 22.33, 22.06, 13.9, 10.36; MS (Fab) m/z 531 $[M]^+$; anal. calcd for $C_{28}H_{50}O_9$ C 63.37 H 9.50; found: C 62.98 H 9.61.

(5′*S*, 8′*R*, 1*S*)-Hexadecane acid-2-hydroxy-2-(2′-isopropyloxy-6′,9′-dioxo-1′,7′-dioxaspiro[4.4]non-8′-yl)-ethylester (9). Acrolein (1 mL, 15 mmol) was added dropwise under nitrogen to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry 2-propanol (40 mL). After stirring for 2 days at 70 °C the mixture was concentrated in vacuo. A colourless residue remained which was recrystallised from 10 mL of 2-propanol. A colourless powder, yield 3.61 g (70%) was obtained: mp 95 °C; IR (KBr) 3426, 2922, 2852, 1844, 1718 cm^{-1}; ^{1}H NMR: δ 5.82 (1s, 1H); 5.54, 5.53 (d, 1H); 5.17, 5.12 (d, 1H); 4.16–3.73 (m, 4H); 2.33–1.08 (m, 38H); 0.87–0.83 (t, 3H); ^{13}C NMR: δ 206.95, 172.87, 172.56, 104.15, 83.12, 79.08, 68.38, 66.66, 63.12, 33.30, 31.07, 31.27, 29.01, 28.86, 28.68, 28.44, 24.27, 23.17, 22.06, 21.19, 13.88; MS (MALDI TOF) m/z 531 $[M+18]^+$; anal. calcd for $C_{28}H_{48}O_8$: C 65.60 H 9.44; found: C 65.57 H 9.12.

(5′*S*, 8′*R*, 1*S*)-Hexadecane acid-2-hydroxy-2-(2′-cyclohexanyloxy-6′,9′-dioxo-1′,7′-dioxaspiro[4.4]non-8′-yl)-ethyl ester (8). Acrolein (1 mL, 15 mmol) was added dropwise under nitrogen to a solution of 6-*O*-palmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry cyclohexanol (40 mL). After stirring for 4 days at 70 C the mixture was concentrated in vacuo to give of a colourless oil. The oil was crystallized from 10 mL of EtOAc. A colourless powder, yield 2.72 g (49%) was obtained: mp 107–115 °C; IR (KBr) 3472, 2922, 2852, 1804, 1770 cm^{-1}; ^{1}H NMR: δ 5.65 (s, 1H); 4.85–4.84 (m, 1H); 4.40–4.13 (m, 4H); 3.64–3.51 (m, 1H); 2.37–1.25 (m, 42H); 0.89–0.86 (t, 3H); ^{13}C NMR: δ 206.12, 173.78, 172.73, 104.75, 82.14, 79.32, 75.35, 68.94, 63.93, 34.81, 34.00 33.29, 31.94, 31.43, 29.62, 29.48, 29.38, 29.28, 29.15, 25.72, 25.47, 24.83, 24.27, 24.09, 22.71, 14.13; MS (Fab) m/z 553 $[M]^+$; anal. calcd for $C_{31}H_{52}O_8$: C 67.48 H 9.32; found: C 67.15 H 9.27.

(5′*S*, 8′*R*, 1*S*)-Bis-hexadecane acid-2-(2′-hydroxy-6′,9′-dioxo-1′,7′-dioxaspiro[4.4]non-8′-yl)-1,2-ethanediol ester (10). Acrolein (1 mL, 15 mmol) was added dropwise under nitrogen to a solution of 5,6-*O*-dipalmitoyl-L-ascorbic acid (4.15 g, 10 mmol) in dry tert-butanol (40 mL). After the mixture was stirred for 4 days at 70 °C it was concentrated in vacuo to give of a colourless oil, 2.87 g (82%): IR (KBr) 3440, 2956, 2918, 2850, 1772, 1746 cm^{-1}; ^{1}H NMR: δ 5.84 (m, 1H); 5.51–5.48 (m, 1H); 5.09–5.06 (m, 1H); 4.45–4.38 (m, 2H); 2.37–1.25 (m, 60H); 0.89–0.86 (t, 6H, $^3J_{H,H}=6.3$ Hz); ^{13}C NMR: δ 205.77, 205.40, 173.33, 173.10, 172.64, 172.20, 172.12, 102.32, 102.01, 81.00, 80.76, 79.11, 78.88, 68.77, 68.72, 60.93, 60.88, 33.93, 31.28, 29.02, 28.98, 28.87, 28.69, 28.45, 24.34, 22.33, 22.06, 14.13; MS (Fab) m/z 710 $[M+1]^+$. Anal. calcd for $C_{41}H_{72}O_9$: C 69.45 H 10.24; found: C 69.34 H 10.38.

Protein phosphatase assays

Alkaline phosphatase (from calf intesinal mucosa, 17 U/μl; Roche Molecular Biochemicals) was monitored at 405 nm by formation of *p*-nitrophenol from *p*-nitrophenylphosphate. Incubations of 1 mL containing 50 mM Tris–HCl (pH 8), 1 mM magnesium acetate, and phosphatase were started by addition of substrate (final concentration 1.35 mM). Assays at 37 °C were stopped by addition of 0.1 mL 13% K_2HPO_4. Reactions were run such that the colour development resulted in extinction in the range of $0.1 < E_{405} < 1.0$. Applicable controls were carried out because non-enzymatic *p*-nitrophenolphosphate hydrolysis increases with time, temperature and increasing alkaline pH values. All tests were carried out in the linear range of time and protein. Activities were calculated using a molar absorption coefficient of 1.75×10^{-4} M^{-1} cm^{-1}. Acid phosphatase (from potato grade I, 60 mU/μl; Roche Molecular Biochemicals) was assayed similarly, yet at pH 3.25 buffered by 50 mM glycine–HCl. Tyrosine phosphatase (Roche Molecular Biochemicals) was also assayed with *p*-nitrophenolphosphate as a substrate at pH 7.5. A recombinant bovine PP1 tagged with a hexa-histidine was expressed in *Escherichia coli* and affinity purified by Ni^{2+}-agarose chromatography[20] PP1 was assayed with

^{32}P-labeled phosphorylase *a* as a substrate as described elsewhere.[21,22] Test substances were dissolved at appropriate concentrations in DMSO and diluted such that the final DMSO concentrations did not exceed 10%. DMSO controls were routinely carried out to exclude unspecific effects.

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