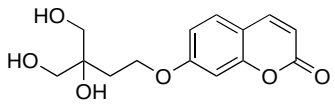
Synthesis and Evaluation of Chromogenic and Fluorogenic Analogs of Glycerol for Enzyme Assays

by **Eva Maria González-García**, **Johann Grognux**, **Denis Wahler**, and **Jean-Louis Reymond***

Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012 Bern
(fax: +41 31 631 80 57; e-mail: jean-louis.reymond@ioc.unibe.ch)

The branched glycerol analogs **1** and **2** were prepared. Mono-ester derivatives of these triols undergo a chromogenic or fluorogenic reaction in the presence of $NaIO_4$. In contrast, both the diesters and the triols are themselves not chromogenic or fluorogenic. Diester derivatives of these triols can be used as probes for lipases. The tris-phosphate derivative of **1** is a fluorogenic substrate for various phosphatases.

Introduction. – Enzyme assays are indispensable tools in enzymology, where they are used to identify enzymes and to evaluate their purity and activity [1]. When an enzyme is discovered, one important question is always the identity of its natural substrate, which is presumably also its best substrate in terms of kinetics behavior. In the context of enzyme assays, the related problem is to find the optimal assay method for any given enzyme, in particular the most-sensitive method. Simple assays giving readily recordable spectroscopic signals are preferred in the context of high-throughput screening for new enzymes by biodiversity mining or directed evolution. Herein, we report the synthesis and evaluation of the branched triols **1** and **2** as building blocks for enzyme substrates. Triols **1** and **2** are close analogs of glycerol, and their ester derivatives can be used to prepare chromogenic and fluorogenic analogs of glycerides, the natural substrates of lipases. The synthesis of these triols and their derivatives and their applications in enzyme assays are presented.



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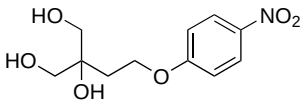

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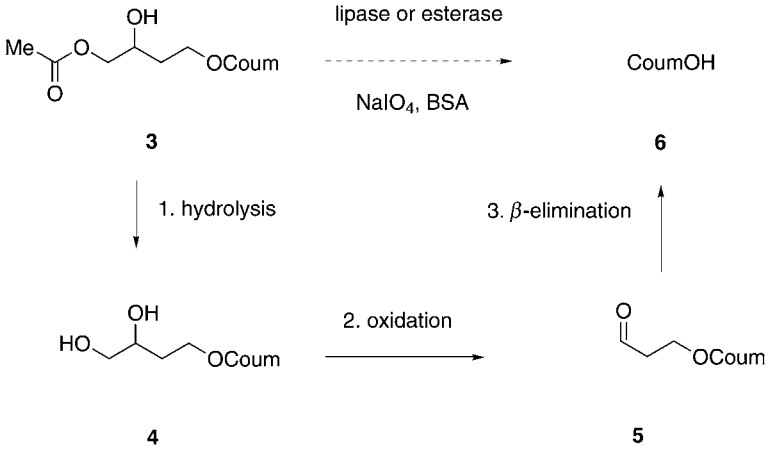
Results and Discussion. – We recently reported a general assay for hydrolytic enzymes based on substrates such as **3**, which release a 1,2-diol such as **4** upon reaction with the enzymes (*Scheme 1*) [2]. The 1,2-diol product then reacts with $NaIO_4$ to generate a labile carbonyl product, *e.g.*, **5**, which subsequently undergoes a β-elimination to give a fluorescent or colored phenol, *e.g.*, umbelliferone **6**, under catalysis by bovine serum albumin (BSA) [3]. The indirect release strategy for the colored/fluorescent products makes these precursors particularly resistant to non-specific hydrolysis. This property can be ascribed to the aliphatic nature of the alcohol leaving group in the enzyme-labile functional group, and provides a key advantage over

the highly labile esters of 4-nitrophenol or umbelliferone (= 7-hydroxy-2*H*-1-benzopyran-2-one), which tend to hydrolyze spontaneously. Although these substrates showed satisfactory reactivity with lipases and esterases, we were intrigued by the possibility to improve their specific reactivities by designing a structure that would be a close analog of glycerol, which is the core element of the natural triglycerides. Triols **1** and **2**, which contain glycerol as part of their branched structures, were, therefore, investigated as building blocks for specific lipase substrates.

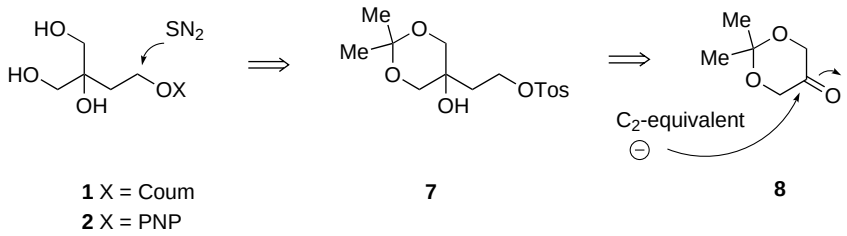
Scheme 1. *Principle of $NaIO_4$ Coupled Enzyme Assay for Hydrolytic Enzymes*



Coum = 2-Oxo-2*H*-1-benzopyran-7-yl

Retrosynthetic analysis suggested to prepare triols **1** and **2** in a divergent synthesis by alkylation of the phenols with the versatile precursor **7** (*Scheme 2*). The branched C-framework in **7** would itself be assembled by addition of a carbon nucleophile to the C=O group of the protected dihydroxyacetone building block **8** to form the tertiary alcohol. This strategy offered a significant simplification over a previously described preparation of intermediate **7** starting with the alkylation of malonate [4].

Scheme 2. *Retrosynthetic Analysis for the Synthesis of Glycerol Analogs*



1 X = Coum
2 X = PNP

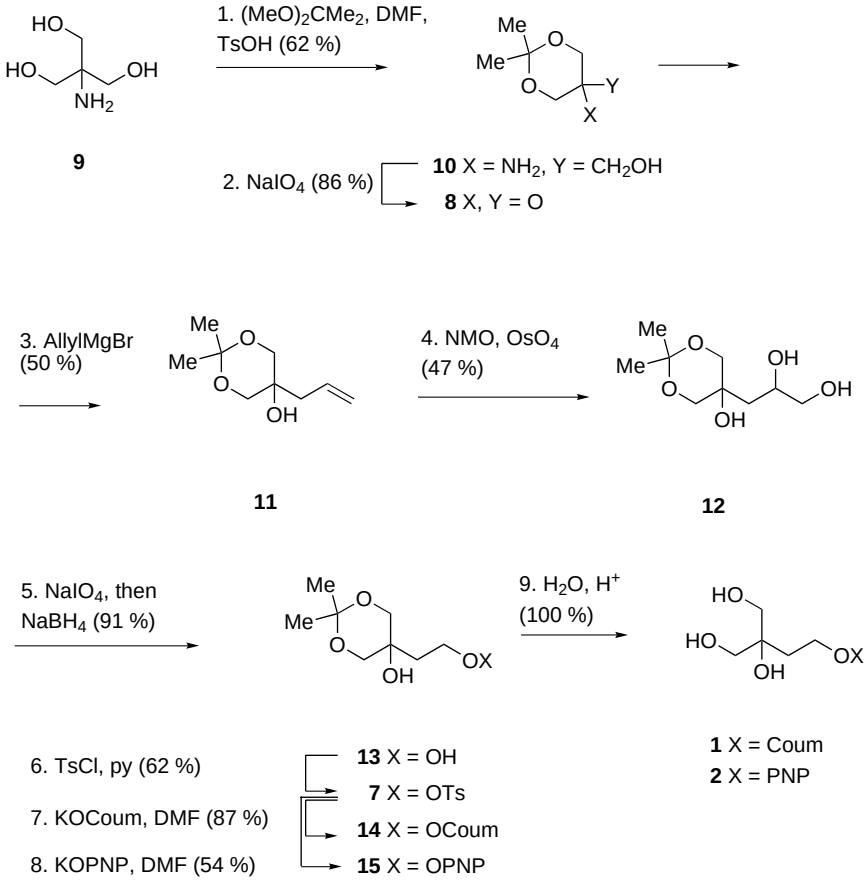
7

8

Protected dihydroxyacetone **8** was obtained from 2-amino-2-(hydroxymethyl)propane-1,3-diol (**9**) by acetonide formation to **10** and oxidation with $NaIO_4$ (*Scheme 3*) [5]. Reaction with allylmagnesium bromide gave the tertiary alcohol **11** in 50% yield. The yield could not be improved despite many modifications of the procedure,

probably due to a competing deprotonation of the ketone by the *Grignard* reagent. While reductive ozonolysis of **11** (O_3, then; $NaBH_4$, or Me_2S and $NaBH_4$, or $(EtO)_3P$ and $NaBH_4$) did not give isolable products, a three-step sequence involving OsO_4-catalyzed dihydroxylation to form diol **12**, oxidative cleavage by $NaIO_4$, and reduction of the resulting aldehyde with $NaBH_4$, gave the desired diol **13**. Selective tosylation of the primary alcohol was achieved with TsCl in pyridine to give the key intermediate **7**. Careful control of the reaction was necessary, as small amounts of the corresponding bis[toluene-4-sulfonate] were, quite surprisingly, also formed. The toluene-4-sulfonate was then substituted with either 4-nitrophenol or umbelliferone to give **1** and **2**, respectively.

Scheme 3. *Synthesis of Triols* **1** *and* **2**

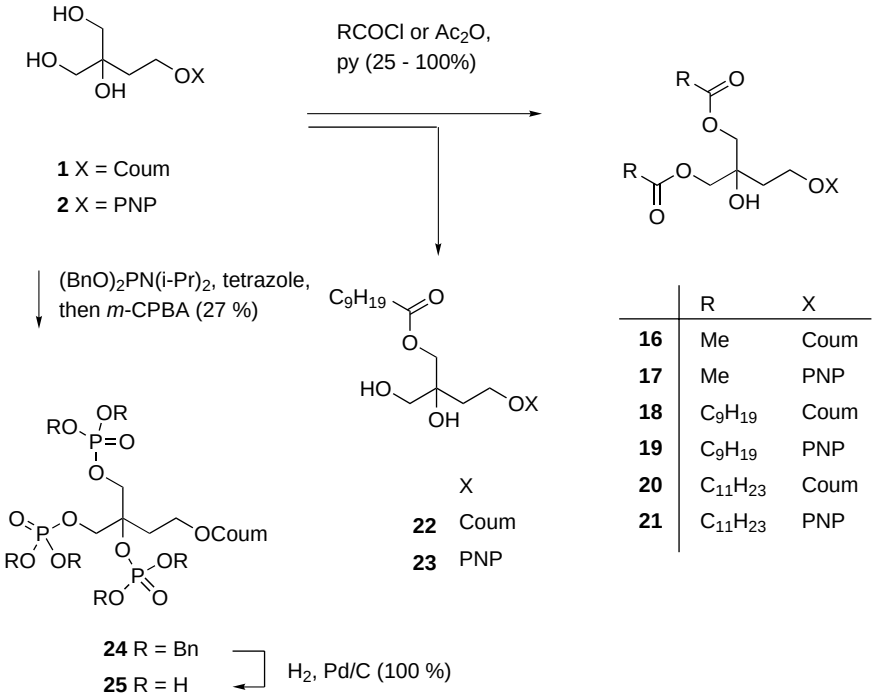


NMO = 4-Methylmorpholine 4-oxide, PNP = 4-nitrophenyl, Coum = 2-oxo-2*H*-benzopyran-7-yl

These triols were then converted to potential probes for enzyme activities (*Scheme 4*). Acetylation gave the diacetates **16** and **17** quantitatively. Reaction with decanoyl chloride or dodecanoyl chloride gave the corresponding diesters **18**–**21**,

which might serve as chromogenic or fluorogenic analogs of glycerides. The monododecanoyl esters **22** and **23** were also prepared. Finally, the tris-phosphate derivative **24** was obtained by reaction with dibenzyl *N*,*N*-diisopropyl phosphoramidite in the presence of 1*H*-tetrazole, followed by oxidation with *m*-chloroperbenzoic acid (*m*-CPBA). Hydrogenolysis effected clean and quantitative removal of the Bn protecting groups to give tris-phosphate **25** as a substrate for phosphatases. It should be noted that the functionalization of the sparingly soluble triols **1** and **2** gave generally low yields despite careful control of the reaction conditions, in particular, drying of the starting triols. Similarly, no products were formed in a series of attempts at monophosphorylation of triols **1** or **2**, or monoesters **22** and **23** according to the published phosphorylation protocols [6] to produce monophosphate or phosphocholine ester derivatives (for the assembly of a phospholipase substrates).

Scheme 4. *Functionalization of Triols* **1** *and* **2** *into Enzyme Substrates*



Coum = 2-Oxo-2*H*-1-benzopyran-7-yl, PNP = 4-nitrophenyl

First, we investigated the reactivity of the triols in aqueous buffer in the presence of $NaIO_4$ and BSA. There was only a very small release of fluorescence or color upon treatment of 0.1 mM aqueous solutions of the triols under our standard conditions (1 mM $NaIO_4$, 2 mg/ml BSA, 20 mM aq. borate pH 8.8 or phosphate pH 7.0). The absence of a fluorogenic/chromogenic reaction with excess $NaIO_4$ can be interpreted in terms of an overoxidation of the intermediate hydroxy ketone **27** by a second C,C-bond cleavage to form acid **28** and a second equiv. of formaldehyde, this overoxidation taking

place faster than the β-elimination induced by BSA (*Scheme 5*). The formation of acid **28** *via* an unstable intermediate was confirmed by analyzing the reaction of triol **1** by HPLC (*Table 1*) and ^{1}H-NMR (see *Exper. Part*). Intrigued by this observation, we also investigated the reactivity of diesters **16**–**21**, mono-esters **22** and **23**, and tris-phosphate **25** in the presence of $NaIO_4$ and BSA. There was no reaction with diesters or triphosphate as expected from the fact that these substrates do not have any unprotected 1,2-diol functionalities. Mono-esters **22** and **23**, which do have the critical 1,2-diol functional groups, reacted with $NaIO_4$ and BSA to give the colored/fluorescent products 4-nitrophenol or umbelliferone. The oxidation–β-elimination sequence proceeded with a half-life of $t_{1/2} = 15$ min, which is comparable to that of simple diols such as **4**.

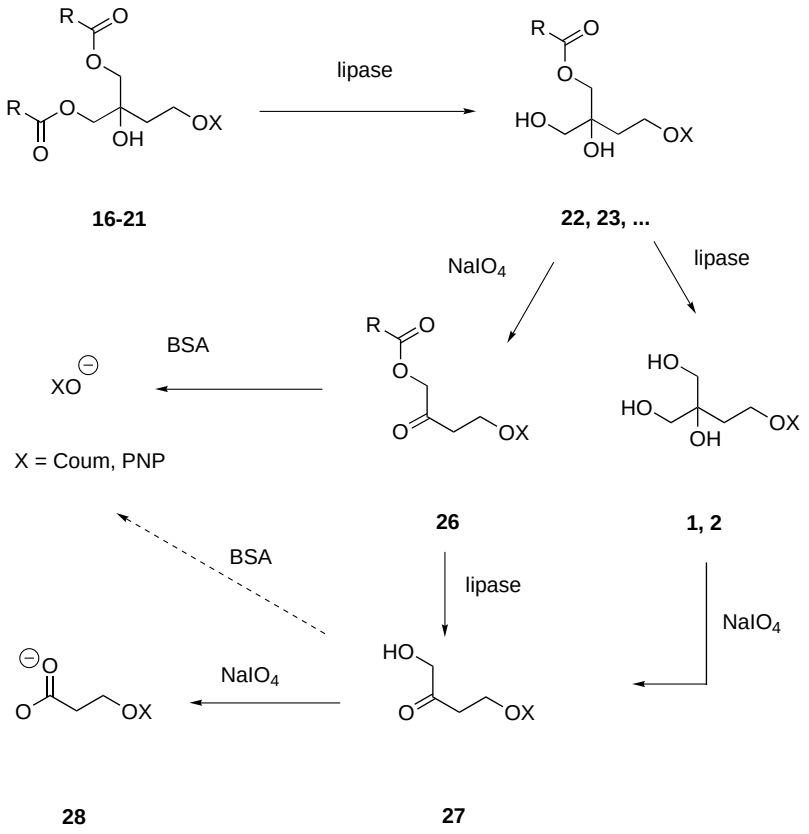
Table 1. *Reaction of Triol* **1** *with* $NaIO_4$ *Analyzed by HPLC*[a])

	t_R 4.8 min (**1**+**8**)	t_R 7.8 min (**27**)	t_R 12.5 min (**28**)
A, $t = 2$ min	63	37	0
A, $t = 1$ h	49	47	4
A, $t = 5$ h	90	7	3
A, $t = 24$ h	96	0	4
B, $t = 2$ min	34	66	0
B, $t = 1$ h	30	43	27
B, $t = 6$ h	25	17	58
B, $t = 24$ h	14	4	82

[a]) The peak area observed are given in % for the reaction of 100 μM triol **1** in aq. 20 mM borate pH 8.8, 2 mg · ml^{-1} BSA, 25°, and *A:* 100 μM $NaIO_4$, *B:* 1 mM $NaIO_4$. Isocratic elution at 1.5 ml · min^{-1} of 88% H_2O, 12% MeCN, 0.1% CF_3COOH; detection by UV at 325 nm; analytical column: *Chromolit Performance RP-18e* (*Merck*), 100 × 4.6 mm (1.02129.0001, Charge No. OB 14306)

We next investigated the mono- and diester derivatives for use as lipase probes. Due to the overoxidation chemistry observed with the triols, one could expect that a chromogenic or fluorescent signal would be obtained only for lipases converting selectively one of the diesters **16**–**21** to a mono-ester. Formation of the mono-ester would be immediately followed by oxidation by $NaIO_4$ to give an acyloxy ketone of type **26** as for the reaction of the mono-esters with $NaIO_4$. At that stage, the β-elimination leading to the spectroscopic signal would take place only when the BSA-catalyzed reaction was faster than a possible further hydrolysis of **26** by the lipase, which would lead to hydroxy ketone **27** and, ultimately, to acid **28**. This reactivity pattern was indeed observed. Three commercial lipases, *Rhizopus arrizus* lipase (RAL), *Pseudomonas fluorescens* lipase (PFL), and *Pseudomonas species type B* lipoprotein lipase (PSBL), were chosen as test enzymes (*Fig.*). The reactions at the lower concentration of enzyme gave generally stronger signals, with the bis-decanoates **18** and **19** giving the best ratios of catalytic rate over background (V_{rel}, in parentheses in *Table 2*). This can be interpreted in terms of the chemistry discussed above, with the enzyme reacting rapidly with the diester substrates, and much more slowly with the acyloxy-ketone intermediate **26**, such that the BSA-catalyzed β-elimination could take place. Excess enzyme induces hydrolysis of **26**, which prevents the formation of umbelliferone or 4-nitrophenol by β-elimination. Any reaction that does not give a

Scheme 5. *Reaction Pathways for the Reaction of Triol* **1** *and* **2**, *and Their Ester Derivatives* **16**–**23** *in Aqueous Buffers Containing* $NaIO_4$, *BSA*, *and Lipases*



significant spectroscopic signal might, therefore, signal either lack of reactivity of the lipase, or very high reactivity leading to the rapid formation of **28**.

The nonchromogenic and nonfluorogenic oxidation of triols **1** and **2**, respectively, with $NaIO_4$ offered, in principle, the opportunity to assay esterification reactions of these substrates according to an endpoint oxidation protocol. The triols would be subjected to an esterification reaction, and the formation of a mono-esterified product would be revealed after some time by oxidation with $NaIO_4$. Various attempts to subject these triols sequentially to a lipase/esterase-catalyzed esterification with vinyl acetate in aqueous or organic phase, followed by reaction with periodate and BSA to reveal the formation of a mono-esterification product, gave no results.

We next investigated the use of tris-phosphate **25** as a fluorogenic probe for phosphatases. This substrate seemed promising as a possible chemoselective probe for phytases against alkaline phosphatase, and as a tool to facilitate the discovery of novel phytases by biodiversity mining. Phytases hydrolyze phytate, which is hexaphosphorylated inositol, and are used to enhance the nutritional value of animal feed formulations

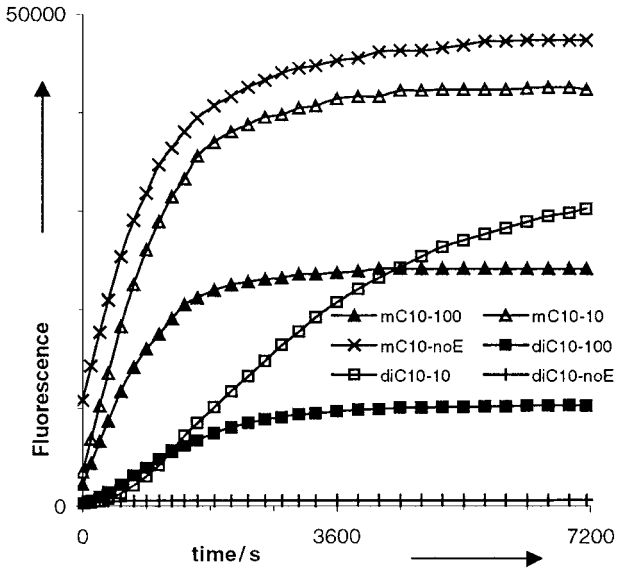


Figure. *Fluorogenic reaction of umbelliferyl ethers* **22** (mC10) *and* **18** (diC10) *with* Rhizopus arrizus *lipase* (*Fluka* No. 62305) *at 100* $\mu g \cdot ml^{-1}$ (100), 10 $\mu g \cdot ml^{-1}$ (10), *or no enzyme* (noE). Conditions: 100 μM substrate in aq. 20 mM borate buffer pH 8.8, 2 $mg \cdot ml^{-1}$ BSA, 1 mM $NaIO_4$, 30°. 0.1-ml Assays were carried out in round-bottom *polypropylene 96* well-plates (*Costar*) and recorded with a *Cytofluor II Fluorescence Plate Reader* (*Perseptive Biosystems*; filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm).

Table 2. *Initial Rates of Formation of Umbelliferone or 4-Nitrophenol from Lipase Substrates*[a])

No.	X	Ester	No Enz.	RAL[b]) 100	RAL[b]) 10	PFL[c]) 100	PFL[c]) 10	PSBL[d]) 100	PSBL[d]) 10
16	Coum	di-C_2	0.38	0.33	0.47	0.53	0.55	*5.3 (13)*	*1.5 (3)*
17	PNP	di-C_2	1.3	1.0	1.1	1.4	1.3	*19 (14)*	*5.3 (3)*
18	Coum	di-C_{10}	0.09	*6.0 (66)*	*13 (143)*	*3.2 (35)*	*9.0 (99)*	0.11	0.15
19	PNP	di-C_{10}	0.58	*5.2 (8)*	*2.8 (4)*	*4.7 (7)*	1.2	0.59	1.2
20	Coum	di-C_{12}	0.10	*0.90 (8)*	*2.2 (21)*	*1.2 (11)*	*1.3 (12)*	*0.49 (4)*	*0.48 (4)*
21	PNP	di-C_{12}	0.11	*0.18 (84)*	*9.3 (4)*	*0.59 (14)*	1.7	0.26	0.32
22	Coum	mono-C_{10}	53	25	50	12	45	0.22	1.8
23	PNP	mono-C_{10}	24	11	27	2.0	4.1	0.57	0.60

[a]) Apparent rate of formation of umbelliferone (**6**) or 4-nitrophenol, in $nM \cdot s^{-1}$. The number in parentheses is the relative reaction rate $V_{rel} = (V_{obs}/V_{no\ enz.}) - 1$. V_{rel} is given only when $V_{rel} > 3$, which indicates the detection limit for catalysis, and these numbers are marked in italics. Conditions: 100 μM substrate in 20 mM aq. borate buffer pH 8.8, 30°, 2 $mg \cdot ml^{-1}$ BSA, 1 mM $NaIO_4$, and a lipase at the indicated concentration in $\mu g \cdot ml^{-1}$. [b]) *Rhizopus arrizus* lipase (RAL), *Fluka* No. 62305. [c]) *Pseudomonas fluorescens* lipase (PFL), *Fluka* No. 62321. [d]) *Pseudomonas species type B* lipoprotein lipase (PSBL), *Fluka* No. F 62336.

by promoting the release of phosphate from phytate, which is abundant in plants. Phytase treatment makes phosphate bio-assimilable by the animal and, thus, prevents the release of the polluting phytate into soil [7]. Due to its polyanionic nature reminiscent of phytate, we expected that tris-phosphate **25** might be selectively cleaved

by phytases only, and might not be recognized by alkaline phosphatase. Tris-phosphate **25** was tested with four different phosphatases at various pH values according to an endpoint protocol, leading, in certain cases, to a fluorogenic reaction (*Table 3*). The occurrence of a fluorogenic reaction implied that two of the three phosphate groups were cleaved upon incubation with the various phosphatases to form a primary monophosphate, which then underwent the fluorogenic oxidation/β-elimination sequence upon treatment with $NaIO_4$ and BSA during the endpoint treatment. Two of the three phytases tested showed significant reactivity with **25** at pH 2.5, under which conditions the reaction with alkaline phosphatase (CIAP) was very slow. However, alkaline phosphatase also reacted with the substrate at pH 5.5, indicating that the observed selectivity at low pH might be more related to the intrinsic pH optima of the different enzymes than to actual substrate selectivity.

Table 3. *Reaction of Triphosphate* **25** *with Various Phosphatases*[a])

Enzyme	pH 2.5	pH 4.5	pH 5.5	pH 6.5
CIAP[b])	1.3	2.2	15.2	1.3
A.f. phytase[c])	58.0	67.0	79.5	0.6
Novo phytase	3.6	5.4	2.7	0.7
Natuphos phytase	26.8	8.0	4.5	0.9

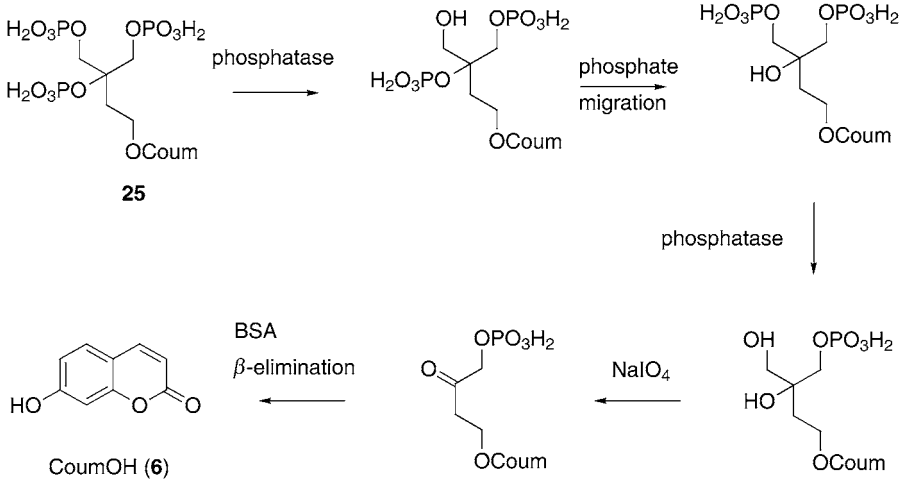
[a]) Amount of umbelliferone (**6**) formed given in percent. Conditions: 0.1 mg · ml^{-1} enzyme, 100 μM substrate **25**, 10 mM aq. citrate, acetate, or phosphate with 1 mM $CaCl_2$, 55°. Enzymes and substrates are incubated for 1 h at the desired pH and 55°, the pH is then adjusted to 7.2 with 0.5M aq. dibasic phosphate. $NaIO_4$ (final conc. 1 mM) and BSA (final conc. 2 mg · ml^{-1}) are added, and the reactions incubated for a further 60 min before fluorescence reading. Fluorescence was converted to umbelliferone (**6**) concentration with a calibration curve. [b]) CIAP: calf intestinal alkaline phosphatase. [c]) *A. f.* phytase: *Aspergillus ficuum* phytase.

The conversion of tris-phosphate **25** to monophosphate **31** probably takes place by hydrolysis of a primary phosphate group, migration of the phosphate group to the primary OH group thus liberated, and further hydrolysis of a primary phosphate group to give a mono-phosphate intermediate capable of undergoing a fluorogenic oxidation/β-elimination sequence (*Scheme 6*). This process could not be investigated in detail due to the limited amount of trisphosphate available.

The favorable reactivity of our tris-phosphate **25** with enzymes, in particular the suitable processing of monophosphate **31** to a fluorescent product, indicated that the triols **1** and **2** might serve as useful probes for kinase reactions. The reaction with glycerol kinase, which phosphorylates glycerol to glycerol 1-phosphate with adenosine triphosphate (ATP), was investigated [8]. Unfortunately, we could not detect any sign of phosphorylation by endpoint treatment with $NaIO_4$ and BSA. The lack of reactivity of glycerol kinase with triols **1** or **2** can be explained by the fact that this enzyme is highly selective for glycerol.

Conclusions. – In summary, glycerol analogs **1** and **2** were prepared by an efficient synthesis starting with 2-amino-2-(hydroxymethyl)propane-1,3-diol (**9**). Bis-decanoates **18** and **19** were found to be reactive probes for lipases under dilute enzyme conditions, and the tris-phosphate **25** operates as a fluorogenic phosphatase substrate, with selectivity for phytases at low pH values.

Scheme 6. *Hypothetical Mechanism for the Dephosphorylation of Triphosphate* **25** *Leading to the Fluorescent Umbelliferone* (**6**). Treatment with $NaIO_4$ and BSA is carried out after incubation with phosphatase enzymes.



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Experimental Part

General. All reactions were monitored by TLC on *Alugram SIL G/UV$_{254}$* silica-gel sheets (*Macherey-Nagel*) with detection by UV or with 0.5% phosphomolybdic acid soln. in 95% EtOH. Silica gel *60* (*Macherey-Nagel* 230–400 mesh) was used for flash chromatography (FC). M.p.: *Kofler* or *Büchi 510* apparatus; uncorrected. IR Spectra: *Perkin-Elmer Spectrum One* series FTIR apparatus. ^{1}H- and ^{13}C-NMR spectra: *Bruker AC-300* spectrometer.

*7-[3,4-Dihydroxy-3-(hydroxymethyl)butoxy]-2*H*-1-benzopyran-2-one* (**1**). A soln. of compound **14** (420 mg, 1.30 mmol) in THF (8 ml) and 1N HCl (8 ml) was stirred for 1 h until TLC indicated disappearance of starting material. The mixture was extracted with AcOEt (10 × 200 ml), dried (Na_2SO_4), filtered, and the solvent was evaporated *in vacuo* to yield **1** (362 mg, 100%). Pale-yellow solid. M.p. 124–126°. TLC (AcOEt): R_f 0.67. IR (KBr): 3437*s* (br.), 1703*s*, 1622*s*, 1034*m*, 847*s*. ^{1}H-NMR (CD_3OD, 300 MHz): 7.86 (*d*, *J* = 8.6, 1 H); 7.52 (*d*, *J* = 8.6, 1 H); 6.95–6.92 (*m*, 2 H); 6.22 (*d*, *J* = 9.6, 1 H); 4.27 (*t*, *J* = 7.0, 2 H); 3.55 (*s*, 4 H); 2.04 (*t*, *J* = 7.0, 2 H). ^{13}C-NMR (CD_3OD, 75 MHz): 163.9; 163.4; 157.1; 145.7; 130.4; 114.2; 114.0; 113.3; 102.3; 74.8; 66.4 (2 C); 65.9; 34.1. EI-MS: 279 (6, $[M-H]^+$), 149 (63), 134 (21), 84 (76), 66 (100).

2-(Hydroxymethyl)-4-[(4-nitrophenyl)oxy]butane-1,2-diol (**2**). A soln. of compound **15** (330 mg, 1.12 mmol) in THF (4 ml) and 1N HCl (4 ml) was stirred for 30 min at r.t. The mixture was extracted with AcOEt (10 × 40 ml). The org. phases were dried (Na_2SO_4), and the solvent was evaporated to provide **2** (288 mg) in a quant. yield. Colorless solid. M.p. 69–71°. TLC (AcOEt): R_f 0.59. IR (KBr): 3393*s* (br.), 1594*s*, 1506*s*, 1343*s*, 1264*s*, 1112*m*. ^{1}H-NMR (CD_3OD, 300 MHz): 8.20 (*d*, *J* = 9.2, 2 H); 7.08 (*d*, *J* = 9.2, 2 H); 4.31 (*t*, *J* = 7.0, 2 H); 3.55 (*s*, 4 H); 2.04 (*t*, *J* = 7.0, 2 H). ^{13}C-NMR (CD_3OD, 75 MHz): 165.4; 142.4; 126.7 (2 C); 115.6 (2 C); 74.7; 66.3 (2 C); 65.9; 34.0. EI-MS: 257 (5, M^+), 226 (67), 208 (59), 152 (77), 140 (83), 101 (96), 71 (100), 43 (82). HR-MS (ESI$^+$-TOF-MS): 258.0979 ($C_{11}H_{16}NO_6^+$, $[M+H]^+$; calc. 258.0977).

2-(5-Hydroxy-2,2-dimethyl-1,3-dioxan-5-yl)ethyl 4-Methylbenzenesulfonate (**7**). TsCl (554 mg, 2.90 mmol) was added to a soln. of diol **13** (465 mg, 2.64 mmol) in anh. pyridine (10 ml) at 0°. The mixture was stirred at 5° for 3 d. The mixture was quenched with H_2O (5 ml) and extracted with AcOEt (3 × 50 ml). The org. phases were dried (Na_2SO_4), and the solvents were evaporated *in vacuo*. The crude product was chromatographed (silica gel; hexane/AcOEt 1:1) to yield **7** (538 mg, 62%). Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.33. IR (film): 3374*m*, 1216*s*, 1187*s*, 1038*m*, 815*m*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.78 (*d*, *J* = 8.2, 2 H); 7.35 (*d*, *J* = 8.2,

2 H); 4.22 (*t*, *J* = 5.9, 2 H); 3.80 (*d*, ^2J = 12.0, 2 H); 3.50 (*d*, ^2J = 12.0, 2 H); 3.22 (br. *s*, 1 H); 2.46 (*s*, 3 H); 1.73 (*t*, *J* = 5.9, 2 H); 1.42 (*s*, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 145.6; 133.5; 130.6 (2 C); 128.6 (2 C); 99.1; 69.1 (2 C); 66.7; 66.3; 33.8; 29.1; 22.3; 19.0. EI-MS: 315 ([*M* – Me]$^+$), 172 (71), 91 (100), 41 (74). HR-MS (ESI$^+$-TOF-MS): 331.120 ($C_{15}H_{23}O_6S^+$, [*M* + H]$^+$; calc. 331.1215).

2,2-Dimethyl-1,3-dioxan-5-one (**8**). A soln. of $NaIO_4$ (8.5 g, 39.9 mmol) in H_2O (110 ml) was added dropwise at 0°, over a period of 3 h, to a slurry of **10** (6.4 g, 39.9 mmol) and KH_2PO_4 (5.4 g, 39.9 mmol) also in H_2O (130 ml). The resulting mixture was stirred at 5° for 1 h and then 5 h at r.t., $Na_2S_2O_3$ (9.9 g, 39.9 mmol) was added, and the resulting soln. was stirred for a further 15 min. Then, the mixture was extracted with CH_2Cl_2 (10 × 50 ml). The combined org. phases were dried (Na_2SO_4), filtered, concentrated *in vacuo*, and purified by bulb-to-bulb distillation (95°) to afford **8** (4.47 g, 86%). Colorless volatile oil. TLC (hexane/AcOEt 1:1): R_f 0.46. EI-MS: 130 (12, M^+), 115 (27), 100 (23), 72 (44), 43 (100). IR (film): 1752. ^{1}H-NMR ($CDCl_3$, 300 MHz): 4.1 (*s*, 4 H); 1.41 (*s*, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 208.7; 100.7; 67.4 (2 C); 24.1 (2 C). EI-HR-MS: 130.062960 ($C_9H_{10}O_3^+$; calc. 130.062994).

5-Amino-2,2-dimethyl-1,3-dioxane-5-methanol (**10**). TsOH (603 mg, 3.17 mmol) was added to a soln. of 2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride (10 g, 63.4 mmol) in dry DMF (70 ml) at r.t., followed by the addition of 2,2-dimethoxypropane (8.55 ml, 69.7 mmol) in one portion. The resulting white suspension became clear and colorless after 30 min. After stirring 12 h, Et_3N (0.5 ml, 36 mmol) was added, and the mixture was stirred for an additional 10 min. The mixture was next concentrated *in vacuo* and treated with Et_3N (7 ml) and AcOEt (250 ml). The white precipitate formed upon addition of the base was collected by filtration. The crude product was recrystallized in Et_2O (7 ml) to afford **10** (6.40 g, 62%). Colorless solid. M.p. 67–69°. TLC (CH_2Cl_2/MeOH 9:1): R_f 0.31. EI-MS: 146 (80, [*M* – Me]$^+$), 130 (85), 73 (100), 42 (73). IR (KBr): 3266.0*s*, 1603.7*s*, 1372.4*s*, 1059.8*s*, 830.0*s*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 3.80 (*d*, ^2J = 12.0, 2 H); 3.54 (*d*, ^2J = 12.0, 2 H); 3.50 (*s*, 2 H); 1.98 (br. *s*, 3 H); 1.46 (*s*, 3 H); 1.42 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 99.1; 67.9 (2 C); 65.6; 50.9; 25.6; 22.8.

2,2-Dimethyl-5-(prop-2-enyl)-1,3-dioxan-5-ol (**11**). Allylmagnesium bromide (*ca.* 1M in hexane, 14.1 ml, 14.15 mmol) was added to a soln. of **8** (920 mg, 7.08 mmol) in dry THF (40 ml) at 0° during 5 min. The mixture was stirred for 2 h at r.t., then the reaction was quenched with 5N NH_4Cl (5 ml), the mixture was extracted with AcOEt (3 × 50 ml) and washed with brine (2 × 50 ml). The org. phases were dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was chromatographed (silica gel; hexane/AcOEt 7:3) to afford **11** (610 mg, 50%). Colorless oil. TLC (hexane/AcOEt 7:3): R_f 0.6. IR (film): 3448*m*, 2994*m*, 1641*w*, 1374*s*, 1200*s*, 830*s*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 5.92–5.78 (*m*, 1 H); 5.14–5.07 (*m*, 2 H); 3.78 (*d*, ^2J = 11.8, 2 H); 3.53 (*d*, ^2J = 11.8, 2 H); 3.17 (br. *s*, 1 H); 2.16 (*d*, *J* = 7.3, 2 H); 1.43 (*s*, 3 H); 1.41 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 132.3; 119.5; 89.9; 69.0 (2 C); 67.2; 39.5; 28.5; 19.6. EI-MS: 171 ([*M* – H]$^+$), 157 (32), 141 (29), 83 (30), 59 (47), 43 (100). HR-MS (ESI$^+$-TOF-MS): 173.1179 ($C_9H_{17}O_3$, [*M* + H]$^+$; calc. 173.1177).

3-(2,2-Dimethyl-5-hydroxy-1,3-dioxan-5-yl)propane-1,2-diol (**12**). 4-Methylmorpholine 4-oxide (1.54 g, 11.36 mmol) and catalytic OsO_4 (0.05 mmol) were added at r.t. to a soln. of **11** (490 mg, 2.84 mmol) in acetone (15 ml) and H_2O (15 ml). The mixture was stirred for 5 h, the reaction was quenched with an aq. soln. of 1N Na_2SO_3 (5 ml), and the mixture was extracted with AcOEt (10 × 100 ml). The combined org. extracts were dried (Na_2SO_4), filtered, and concentrated *in vacuo*, to produce a white precipitate, which was collected by filtration and washed with hexane/AcOEt 1:1, affording **12** (278 mg, 47%). Colorless solid. M.p. 139–141°. TLC (AcOEt): R_f 0.38. IR (KBr): 3393*w*, 2996*m*, 2521*s*, 1730*s*, 1292*s*, 1074*s*. ^{1}H-NMR (CD_3OD, 300 MHz): 3.97–3.90 (*m*, 1 H); 3.83 (*dd*, *J* = 2.2, 11.9, 2 H); 3.64 (2*dd*, *J* = 2.2, 11.9, 2 H); 3.44 (*d*, *J* = 5.5, 2 H); 1.67 (*dd*, *J* = 2.6, 14.7, 1 H); 1.49 (*dd*, *J* = 9.6, 14.7, 1 H); 1.40 (*s*, 6 H). ^{13}C-NMR (CD_3OD, 75 MHz): 99.4; 69.6; 69.4; 69.3; 67.9; 66.4; 38.9; 25.7; 22.0. Anal. calc. for $C_9H_{18}O_5$ (206.24): C 52.41, H 8.80; found: C 52.33, H 8.77.

5-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxan-5-ol (**13**). $NaIO_4$ (303 mg, 1.42 mmol) was added to a soln. of **12** (278 mg, 1.35 mmol) in H_2O (10 ml) at 0°, the mixture was stirred at this temp. for 30 min until the TLC showed that the starting material had disappeared. $NaBH_4$ (64 mg, 1.69 mmol) was added directly, and the mixture was stirred for further 45 min, extracted with AcOEt (5 × 25 ml), dried (Na_2SO_4), filtered, and concentrated *in vacuo*. The product was chromatographed (silica gel; AcOEt/hexane 4:1) to afford **13** (270 mg, 91%). Pale-yellow oil. TLC (AcOEt): R_f 0.53. IR (film): 3415*s*, 2922*s*, 1376*m*, 1200*m*, 1077*s*, 829*m*. ^{1}H-NMR (CD_3OD, 300 MHz): 3.80 (*d*, ^2J = 11.8, 2 H); 3.74 (*t*, *J* = 6.6, 2 H); 3.60 (*d*, ^2J = 11.8, 2 H); 3.50 (br. *s*, 2 H); 1.69 (*t*, *J* = 6.6, 2 H); 1.40 (*s*, 6 H). ^{13}C-NMR (CD_3OD, 75 MHz): 99.3; 69.3 (2 C); 67.8; 58.1; 38.2; 25.6; 22.0. EI-MS: 174 ([*M* – 2 H]$^+$), 145 (34), 72 (76), 70 (46), 59 (100).

*7-[2-(5-Hydroxy-2,2-dimethyl-1,3-dioxan-5-yl)ethoxy]-2*H*-1-benzopyran-2-one* (**14**). To a suspension of *7-hydroxy-2*H*-1-benzopyran-2-one* (**6**; 97.5 mg, 0.6 mmol) in DMF (2 ml), oven-dried K_2CO_3 (167 mg, 1.21 mmol), and 18-crown-6 ether as catalyst, previously stirred for 1 h at r.t., was added a solution of **7**

(200 mg, 0.6 mmol) in DMF (2 ml). The slurry was stirred for 12 h at 95°. After quenching with H_2O (5 ml), the mixture was extracted with AcOEt (3 × 20 ml), washed with aq. NaOH (3 × 20 ml) and brine (3 × 20 ml). The org. phases were dried (Na_2SO_4), filtered, concentrated *in vacuo*, and purified by chromatography to afford **14** (167 mg, 87%). Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.22. IR (film): 3452*m*, 1729*s*, 1614*s*, 1230*m*, 1199*m*, 1126*m*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.63 (*d*, *J* = 9.2, 1 H); 7.36 (*d*, *J* = 9.2, 1 H); 6.83–6.80 (*m*, 2 H); 6.24 (*d*, *J* = 9.6, 1 H); 4.24 (*t*, *J* = 5.9, 2 H); 3.90 (*d*, ^2J = 11.9, 2 H); 3.65 (*d*, ^2J = 11.9, 2 H); 3.39 (br. *s*, 1 H); 1.88 (*t*, *J* = 5.9, 2 H); 1.46 (*s*, 3 H); 1.45 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 162.3; 162.0; 156.6; 143.9; 129.5; 114.0; 113.5; 113.4; 102.3; 99.1; 69.4 (2 C); 67.1; 64.3; 33.8; 28.9; 19.4. EI-MS: 321 (9, $[M-Me]^+$), 320 (33, $[M-H_2O]^+$), 175 (81), 162 (100), 134 (60), 43 (73).

2,2-Dimethyl-5-[2-(4-nitrophenyloxy)ethyl]-1,3-dioxan-5-ol (**15**). A soln. of K_2CO_3 (234 mg, 1.7 mmol), 4-nitrophenol (142 mg, 1.02 mmol), and 18-crown-6 ether (as catalyst, 0.34 mmol) in acetone (6 ml) was stirred for 40 min ar r.t. Compound **7** (280 mg, 0.85 mmol) was added, and the mixture was refluxed overnight. After quenching with H_2O (10 ml) and extraction with AcOEt (3 × 30 ml), the org. phases were dried (Na_2SO_4), and the solvents were evaporated *in vacuo*. The crude product was chromatographed (silica gel; hexane/AcOEt 7:3) to afford **15** (137 mg, 54%). Yellow solid. M.p. 95–97°. TLC (hexane/AcOEt 1:1): R_f 0.59. IR (KBr): 3343*m*, 1606*m*, 1594*s*, 1508*s*, 1341*s*, 1259*s*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 8.20 (*d*, *J* = 9.2, 2 H); 6.95 (*d*, *J* = 9.2, 2 H); 4.29 (*t*, *J* = 5.9, 2 H); 3.91 (*d*, ^2J = 11.7, 2 H); 3.65 (*d*, ^2J = 11.7, 2 H); 3.35 (br. *s*, 1 H); 1.89 (*t*, *J* = 5.9, 2 H); 1.48 (*s*, 3 H); 1.46 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 164.0; 142.2; 126.5 (2 C); 115.0 (2 C); 90.0; 69.2 (2 C); 66.9; 64.4; 33.7; 28.8; 19.2. EI-MS: 298 (48, $[M+1]^+$), 282 (48, $[M-Me]^+$), 240 (100), 152 (35), 119 (35). HR-MS (ESI$^+$-TOF-MS): 298.1299 ($C_{14}H_{20}NO_6^+$, $[M+H]^+$; calc. 298.1290).

*2-(Acetoxymethyl)-2-hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Acetate* (**16**). Ac_2O (1 ml) was added to a soln. of **1** (20 mg, 0.08 mol) in dry pyridine (1 ml), the mixture was stirred overnight at r.t. Then the solvent was evaporated to give **16** in quant. yield. Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.24. EI-MS: 365 (100, $[M+1]^+$), 305 (17), 245 (33), 155 (37), 135 (27), 119 (80). IR (film): 3453*w*, 1733*s*, 1515*s*, 1232*s*, 1127*m*, 1046*m*, 838*w*. ^{1}H-NMR (CD_3OD, 300 MHz): 7.64 (*d*, *J* = 9.5, 1 H); 7.37 (*d*, *J* = 9.4, 1 H); 6.84–6.82 (*m*, 2 H); 6.25 (*d*, *J* = 9.4, 1 H); 4.26 (*t*, *J* = 5.9, 2 H); 4.21–4.12 (*m*, 4 H); 2.90 (br. *s*, 1 H); 2.14–2.06 (*m*, 2 H); 2.11 (*s*, 6 H). ^{13}C-NMR (CD_3OD, 75 MHz): 171.5 (2 C); 162.2; 161.7; 156.5; 143.9; 129.5; 114.0; 113.6; 113.5; 102.1; 72.6; 67.5 (2 C); 64.5; 34.2; 21.5 (2 C). HR-MS (ESI$^+$-TOF-MS): 365.1267 ($C_{18}H_{21}NO_8^+$, $[M+H]^+$; 365.136).

2-(Acetoxymethyl)-2-hydroxy-4-[(4-nitrophenyl)oxy]butyl Acetate (**17**). Ac_2O (1 ml) was added at 0° to a soln. of **2** (20 mg, 0.08 mmol) in dry pyridine (1 ml), and the mixture was stirred overnight at r.t. The solvent was evaporated to give **17** in quant. yield. Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.42. IR (film): 3483*w*, 1740*s*, 1594*s*, 1515*s*, 1342*s*, 1259*s*, 1112*m*. ^{1}H-NMR (CD_3OD, 300 MHz): 8.20 (*d*, *J* = 9.2, 2 H); 6.95 (*d*, *J* = 9.2, 2 H); 4.29 (*t*, *J* = 6.2, 2 H); 4.21–4.12 (*m*, 4 H); 2.14–2.04 (*m*, 2 H); 2.10 (*s*, 6 H). ^{13}C-NMR (CD_3OD, 75 MHz): 171.5 (2 C); 163.9; 142.4; 126.6 (2 C); 115.1 (2 C); 72.5; 67.4 (2 C); 64.7; 34.1; 21.5 (2 C). EI-MS: 341 (15, M^+), 324 (96), 282 (25), 222 (42), 155 (40), 135 (47), 119 (100). HR-MS (ESI$^+$-TOF-MS): 341.11110 ($C_{15}H_{19}NO_8^+$, $[M+H]^+$; calc. 341.11106).

*2-[(Decanoyloxy)methyl]-2-hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Decanoate* (**18**). Decanoyl chloride (182 µl, 0.89 mmol) was added to a soln. of **1** (100 mg, 0.36 mmol) in dry pyridine (6.6 ml) at 0°, and the mixture was stirred at 5° overnight. The solvent was evaporated, and the crude product was extracted with AcOEt (3 × 25 ml), washed with aq. $NaHCO_3$ (3 × 20 ml), and dried (Na_2SO_4). The yellow oil obtained after evaporation of the solvents was chromatographed (silica gel; hexane/AcOEt 4:1) to yield **18** (96 mg, 43%). Colorless crystals. M.p. 64–66°. TLC (hexane/AcOEt 3:2): R_f 0.45. IR (KBr): 3410*m*, 2925*s*, 2855*s*, 1733*s*, 1615*m*, 1470*m*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.64 (*d*, *J* = 9.3, 1 H); 7.38 (*d*, *J* = 9.3, 1 H); 6.95–6.93 (*m*, 2 H); 6.27 (*d*, *J* = 9.3, 1 H); 4.26 (*t*, *J* = 6.5, 2 H); 4.21–4.11 (*m*, 4 H); 2.82 (br. *s*, 1 H); 2.35 (*t*, *J* = 7.7, 4 H); 2.11 (*t*, *J* = 6.5, 2 H); 1.66–1.61 (*m*, 4 H); 1.39–1.20 (*m*, 24 H); 0.88 (*t*, *J* = 6.9, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 174.3 (2 C); 162.2; 161.7; 156.5; 143.9; 129.5; 114.0; 113.9; 113.5; 102.1; 72.7; 67.3 (2 C); 64.6; 34.8 (2 C); 34.2; 32.5 (2 C); 30.2 (2 C); 30.1 (2 C); 30.0 (2 C); 29.9 (2 C); 29.8 (4 C); 25.6 (2 C); 23.3 (2 C); 14.7 (2 C). FAB-MS: 571 (45), 435 (94), 417 (33), 281 (100), 263 (63), 163 (76). Anal. calc. for $C_{34}H_{52}O_8$ (588.77): C 69.36, H 8.90; found: C 69.25, H 8.91.

2-[(Decanoyloxy)methyl]-2-hydroxy-4-[(4-nitrophenyl)oxy]butyl Decanoate (**19**). Decanoyl chloride (157 µl, 0.77 mmol) was added to a soln. of **2** (67 mg, 0.29 mmol) in dry pyridine (4 ml) at 0°, and the mixture was stirred at 5° overnight. The solvent was evaporated, and the crude product was extracted with AcOEt (3 × 15 ml), washed with aq. sat. $NaHCO_3$ (3 × 10 ml), and dried (Na_2SO_4). The crude product obtained after evaporation of the solvents was chromatographed (silica gel; hexane/AcOEt 85:15) to give **19** (63 mg, 38%). Colorless oil. TLC (hexane/AcOEt 7:3): R_f 0.64. IR (film): 3448*w*, 2927*s*, 2856*s*, 2856*s*, 1742*s*, 1594*m*, 1516*m*,

1342*s*, 1262*s*, 1111*m*, 847*w*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 8.19 (*d*, *J* = 9.2, 2 H); 6.95 (*d*, *J* = 9.2, 2 H); 4.28 (*t*, *J* = 6.5, 2 H); 4.21 – 4.10 (*m*, 4 H); 2.85 (br. *s*, 1 H); 2.34 (*t*, *J* = 7.3, 4 H); 2.10 (*t*, *J* = 6.5, 2 H); 1.64 – 1.60 (*m*, 4 H); 1.38 – 1.28 (*m*, 24 H); 0.87 (*t*, *J* = 6.9, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 174.4 (2 C); 164.0; 142.4; 126.6 (2 C); 115.1 (2 C); 72.7; 67.3 (2 C); 64.7; 34.8 (2 C); 34.2; 32.5 (2 C); 30.0 (2 C); 29.9 (4 C); 29.8 (2 C); 25.5 (2 C); 23.3 (2 C); 14.7 (2 C). EI-MS: 565 (6, M^+), 535 (63), 380 (85), 155 (100), 83 (67). HR-MS (ESI$^+$-TOF-MS): 548.3576 ($C_{31}H_{50}NO_7^+$, $[M - H_2 + H]^+$; 548.3587).

*2-[(Dodecanoyloxy)methyl]-2-hydroxy-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Dodecanoate* (**20**). Dodecanoyl chloride (211 µl, 0.89 mmol) was added to a soln. of **2** (100 mg, 0.36 mmol) in dry pyridine (6.6 ml) at 0°, and the mixture was stirred at 5° overnight. The solvents were evaporated, and the crude product was extracted with AcOEt (3 × 25 ml), washed with aq. $NaHCO_3$ (3 × 20 ml), and dried (Na_2SO_4). The yellow oil obtained was chromatographed (silica gel; hexane/AcOEt 4:1). The crude product obtained was further purified by recrystallization from hexane to afford **20** (55 mg, 25%). Colorless crystals. M.p. 69 – 71°. TLC (hexane/AcOEt 3:2): R_f 0.45. IR (KBr): 2958*m*, 2922*m*, 2853*m*, 1738*s*, 1621*m*, 1470*m*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.63 (*d*, *J* = 9.4, 1 H); 7.36 (*d*, *J* = 9.4, 1 H); 6.83 – 6.81 (*m*, 2 H); 6.25 (*d*, *J* = 9.5, 1 H); 4.26 (*t*, *J* = 5.9, 2 H); 4.21 – 4.11 (*m*, 4 H); 2.91 (br. *s*, 1 H); 2.34 (*t*, *J* = 7.4, 4 H); 2.10 (*t*, *J* = 5.9, 2 H); 1.64 – 1.59 (*m*, 4 H); 1.39 – 1.23 (*m*, 32 H); 0.87 (*t*, *J* = 6.6, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 174.3 (2 C); 162.2; 161.7; 156.5; 143.9; 129.5; 114.0; 113.5; 113.4; 102.1; 72.7; 67.3 (2 C); 64.6; 34.8 (2 C); 34.2; 32.5 (2 C); 30.2 (2 C); 30.1 (2 C); 30.0 (2 C); 29.9 (2 C); 29.8 (4 C); 25.6 (2 C); 23.3 (2 C); 14.7 (2 C). Anal. calc. for $C_{38}H_{60}O_8$ (644.88): C 70.77, H 9.38; found: C 70.68, H 9.37.

2-[(Dodecanoyloxy)methyl]-2-hydroxy-4-[(4-nitrophenyl)oxy]butyl Dodecanoate (**21**). Dodecanoyl chloride (161 µl, 0.68 mmol) was added to a soln. of **2** (67 mg, 0.26 mmol) in dry pyridine (4 ml) at 0°, and the mixture was stirred at 5° overnight. The solvent was evaporated, and the crude product was extracted with AcOEt (3 × 15 ml), washed with aq. sat. $NaHCO_3$ (3 × 10 ml), and dried (Na_2SO_4). The crude residue obtained after evaporation of the solvent was chromatographed (silica gel, hexane/AcOEt 85:15) to provide **21** (67 mg, 41%). Colorless oil. TLC (hexane/AcOEt 7:3): R_f 0.79. IR (film): 3381*w*, 2956*s*, 2921*s*, 2852*s*, 1711*s*, 1593*m*, 1343*s*, 1265.5*s*, 1111*m*, 849*w*. ^{1}H-NMR ($CDCl_3$, 300 MHz): 8.20 (*d*, *J* = 9.2, 2 H); 6.95 (*d*, *J* = 9.2, 2 H); 4.29 (*t*, *J* = 6.0, 2 H); 4.21 – 4.11 (*m*, 4 H); 2.81 (br. *s*, 1 H); 2.35 (*t*, *J* = 7.4, 4 H); 2.10 (*t*, *J* = 6.0, 2 H); 1.65 – 1.60 (*t*, *J* = 7.0, 4 H); 1.38 – 1.28 (*m*, 32 H); 0.88 (*t*, *J* = 6.2, 6 H). ^{13}C-NMR ($CDCl_3$, 75 MHz): 174.4 (2 C); 164.0; 142.4; 126.6 (2 C); 115.1 (2 C); 72.7; 67.3 (2 C); 64.7; 34.8 (2 C); 34.2; 32.6 (2 C); 30.2 (4 C); 30.1 (2 C); 30.0 (2 C); 29.9 (2 C); 29.8 (2 C); 25.6 (2 C); 23.2 (2 C); 14.7 (2 C). EI-MS: 621 (7, M^+), 603 (63), 408 (11), 183 (79), 83 (100), 57 (59). Anal. calc. for $C_{35}H_{59}NO_8$ (621.84): C 67.60, H 9.56; found: C 67.72, H 9.55.

*2-Hydroxy-2-(hydroxymethyl)-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butyl Decanoate* (**22**). Decanoyl chloride (17.5 µl, 0.085 mmol) was added to a soln. of **1** (20 mg, 0.071 mmol) in dry pyridine (2 ml) at 0°, and the mixture was stirred overnight at 5°. The solvent was evaporated, and the crude product was extracted with AcOEt (3 × 15 ml), washed with aq. sat. $NaHCO_3$ (3 × 10 ml), and dried (Na_2SO_4). The crude residue obtained after evaporation of the solvents was chromatographed (silica gel; hexane/AcOEt 7:3) to yield **22** (15 mg, 49%). Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.15. IR (film): 3447.4*s*, 2927.3*s*, 2856.6*s*, 1733.5*s*, 1615.8*s*, 1282.3*s*. ^{1}H-NMR (CD_3OD, 300 MHz): 7.64 (*d*, *J* = 9.4, 1 H); 7.38 (*d*, *J* = 9.4, 1 H); 6.85 – 6.83 (*m*, 2 H); 6.26 (*d*, *J* = 9.4, 1 H); 4.27 (*t*, *J* = 5.9, 2 H); 4.23 – 4.16 (*m*, 2 H); 3.60 – 3.52 (*m*, 2 H); 2.86 (br. *s*, 1 H); 2.36 (*t*, *J* = 7.3, 2 H); 2.07 (*t*, *J* = 5.9, 2 H); 1.65 – 1.61 (*m*, 2 H); 1.37 – 1.27 (*m*, 12 H); 0.88 (*t*, *J* = 7.0, 3 H). ^{13}C-NMR (CD_3OD, 75 MHz): 175.3; 163.7; 163.3; 157.1; 145.8; 130.4; 114.2; 114.0; 113.3; 102.3; 73.6; 67.6; 66.4; 65.4; 35.0; 34.4; 33.0; 30.6; 30.4; 30.3; 30.2; 26.0; 23.7; 14.4. EI-MS: 435 (57, M^+); 281 (100); 163 (71). HR-MS (ESI$^+$-TOF-MS): 435.2372 ($C_4H_{35}O_7^+$, $M + H]^+$; calc. 435.2382.

2-Hydroxy-2-(hydroxymethyl)-4-[(4-nitrophenyl)oxy]butyl Decanoate (**23**). Decanoyl chloride (91 µl, 0.44 mmol) was added to a soln. of **2** (105 mg, 0.37 mmol) in dry pyridine (10 ml) at 0°, and the mixture was stirred at 5° overnight. The solvent was evaporated, and the crude product was extracted with AcOEt (3 × 15 ml), washed with aq. sat. $NaHCO_3$ (3 × 10 ml), and dried (Na_2SO_4). The crude residue obtained after evaporation of the solvents *in vacuo* was chromatographed (silica gel; hexane/AcOEt 7:3) to give **23** (64 mg, 42%). Pale-yellow oil. TLC (hexane/AcOEt 1:1): R_f 0.41. IR (film): 3449*m*, 2927*s*, 2856*s*, 1733*s*, 1515*s*, 1262*s*, 1111*s*. ^{1}H-NMR (CD_3OD, 300 MHz): 8.20 (*d*, *J* = 9.2, 2 H); 7.07 (*d*, *J* = 9.2, 2 H); 4.30 (*t*, *J* = 6.6, 2 H); 4.12 (*s*, 2 H); 3.54 (*s*, 2 H); 2.33 (*t*, *J* = 7.7, 2 H); 2.07 (*t*, *J* = 6.6, 2 H); 1.59 – 1.57 (*m*, 2 H); 1.38 – 1.28 (*m*, 12 H); 0.88 (*t*, *J* = 6.9, 3 H). ^{13}C-NMR (CD_3OD, 75 MHz): 175.3; 165.4; 142.8; 126.8 (2 C); 115.8 (2 C); 73.6; 67.7; 66.4; 65.7; 35.0; 34.4; 33.0; 30.6; 30.4 (2 C); 30.3; 30.2; 26.0; 23.7; 14.4. Anal. calc. for $C_{21}H_{33}NO_7$ (411.49): C 61.30, H 8.08; found: C 61.36, H 8.10.

*2-[(Dibenzyloxyphosphoryloxy)methyl]-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Bis(dibenzylphosphate)* (**24**). A soln. of **1** (0.050 g, 0.18 mmol) and 1*H*-tetrazole (0.113 g, 9 equiv.) in anh. CH_2Cl_2

(5 ml) and DMF (2 ml) was treated at 25° under stirring with dibenzyl *N*,*N*-diisopropylphosphoramidite (0.180 ml, 3 equiv.) and stirred at 25° for 3 h. The soln. was cooled to −78°, *m*-CPBA (0.250 g, 6 equiv.) was added, and stirring was prolonged for 1 h at 0°. A soln. of sat. aq. $NaHCO_3$ (1 ml) was added, and the mixture was concentrated *in vacuo*. The residue was taken in AcOEt, washed (sat. aq. $NaHCO_3$, H_2O, and brine) and purified by FC (CH_2Cl_2/MeCN 75:25) to give pure **24** (0.052 g, 27%). Yellow syrup. TLC (CH_2Cl_2/MeCN 90:10, UV 365 nm): R_f 0.43. IR (KBr): 3447*s*, 2092*w*, 1732*s*, 1615*s*, 1558*w*, 1509*w*, 1499*w*, 1457*w*, 1399*w*, 1279*s*, 1016*s*. ^{1}H-NMR (300 MHz, $CDCl_3$): 7.60 (*d*, *J* = 9.6, 1 H); 7.35–7.23 (*m*, 31 H); 6.71–6.59 (*m*, 2 H); 6.25 (*d*, *J* = 9.6, 1 H); 5.00–4.93 (*m*, 12 H); 4.30 (*dd*, *J* = 4.5, 10.7, 2 H); 4.23 (*dd*, *J* = 4.5, 10.7, 2 H); 3.94 (*t*, *J* = 5.9, 2 H); 2.22 (*t*, *J* = 5.9, 2 H). ^{13}C-NMR (75 MHz, $CDCl_3$): 162.1; 161.7; 156.4; 143.9; 136.3; 136.2; 129.5; 129.4; 129.3; 128.9; 128.7; 128.6; 114.0; 113.5; 102.1; 83.9; 70.3; 70.2; 67.7; 63.7; 32.5. ^{31}P-NMR (81 MHz, $CDCl_3$): −0.4; −5.0.

*2-[(Dihydroxyphosphoryloxy)methyl]-4-[(2-oxo-2*H*-1-benzopyran-7-yl)oxy]butane-1,2-diyl Bis(dihydrogenphosphate)* (**25**). A soln. of **24** (0.040 g, 0.038 mmol) in EtOH/H_2O 4:1 (10 ml) was treated with Pd on charcoal (0.002 g, 0.1 equiv.) at 25° under 1 atm H_2 and stirred vigorously for 3 h. Filtration over *Celite* and concentration *in vacuo* gave pure **25** (0.019 g, quant.). Yellow syrup. IR (KBr): 3438*s*, 2929*w*, 2346*w*, 1702*m*, 1619*s*, 1557*w*, 1509*w*, 1385*m*, 1084*s*. ^{1}H-NMR (300 MHz, D_2O/CD_3CN): 7.85 (*d*, *J* = 9.4, 1 H); 7.50 (*d*, *J* = 7.4, 1 H); 6.93 (*m*, 2 H); 6.21 (*d*, *J* = 9.4, 1 H); 4.28 (*m*, 6 H); 2.25–2.15 (*m*, 2 H). ^{13}C-NMR (75 MHz, D_2O/CD_3CN): 164.4; 162.9; 156.3; 146.3; 130.5; 114.2; 113.8; 112.9; 102.5; 82.6; 66.9; 65.5; 64.9; 32.7. ^{31}P-NMR (81 MHz, D_2O/CD_3CN): 3.1; −1.4.

Kinetics Measurements. All substrates were diluted from stock solns. in 50% aq. MeCN and stored at +4°. Enzymes were diluted from 1 mg·ml^{-1} stock solns. of the supplied solid in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4). Assays (0.1 ml) were followed in individual wells of round-bottom polypropylene *96*-well-plates (*Costar*) with a *Cytofluor II Fluorescence Plate Reader* (*Perseptive Biosystems*, filters $\lambda_{ex} = 360 \pm 20$, $\lambda_{em} = 460 \pm 20$ nm), or of polystyrene *96*-well-plates (*Costar*) with a *Spectramax 250 Microplate Spectrophotometer* (*Molecular Devices*). Fluorescence data were converted to umbelliferone or nitrophenol concentration by means of a calibration curve. The rates indicated in *Table 2* are derived from the linear portion in each curve.

Oxidation of **1** *with* $NaIO_4$. A soln. of triol **1** (8.2 mg, 29.3 μmol, 1 equiv.) in MeCN/H_2O 1:1 (35 ml) was stirred at 25° with 10 equiv. of $NaIO_4$ (62.6 mg, 292.6 μmol) for 24 h. The mixture was then lyophilized to give a crude product containing acid **28** (87%) and umbelliferone **8** (13%) as analyzed by HPLC (for conditions, see *Table 1*) and ^{1}H-NMR integration.

Data of **28**: ^{1}H-NMR (300 MHz, (D_6)DMSO): 7.99 (*d*, *J* = 9.2, 1 H); 7.63 (*d*, *J* = 8.8, 1 H); 7.00 (*d*, *J* = 2.6, 1 H); 6.94 (*dd*, *J* = 8.8, 2.6, 1 H); 6.29 (*d*, *J* = 9.2, 1 H); 4.27 (*t*, *J* = 5.9, 2 H); 2.72 (*t*, *J* = 5.89, 2 H). ^{13}C-NMR (100 MHz, (D_6)DMSO): 172.10; 161.50; 160.30; 155.37 (2 C); 144.34; 129.55; 112.64; 112.57; 101.21; 64.45; 33.89. ^{13}C-NMR (DEPT-135; 100 MHz, (D_6)DMSO): 144.09 (CH); 129.30 (CH); 112.39 (CH); 112.32 (CH); 100.96 (CH); 64.20 (CH_2); 33.63 (CH_2). EI-MS: 234 (M^+).

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