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The Substrate Specificity of Tocopherol Cyclase

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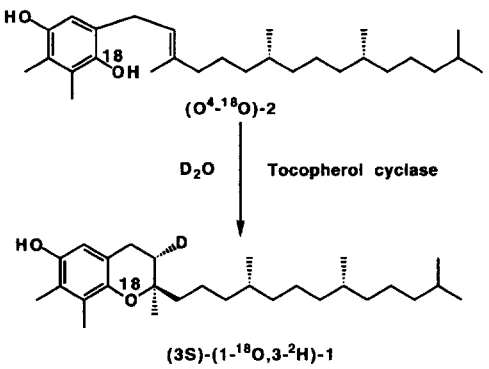
Abstract—The substrate specificity of the enzyme tocopherol cyclase from the blue–green algae *Anabaena variabilis* (*Cyanobacteria*) was investigated with 11 substrate analogues revealing the significance of three major recognition sites: (i) the OH group at C(1) of the hydroquinone, (ii) the (*E*) configuration of the double bond, and (iii) the length of the lipophilic side chain. Experiments with two affinity matrices suggest that substrates approach the enzyme's active site with the hydrophobic tail.

Introduction

Recently, we identified a new enzyme in the blue–green algae *Anabaena variabilis* KUETZING (*Cyanobacteria*) that catalyzes the formation of γ-tocopherol **1** from the phytyl-hydroquinone **2**, Scheme 1. It was shown that the cyclization was stereospecific and could be driven to quantitative substrate turnover under strictly defined conditions such as incubating the 2,6-di-*O*-methyl-β-cyclodextrin complex of **2** in the presence of ascorbic acid with spheroplasts prepared by lysozyme treatment of intact *Anabaena* cells.[1]



Scheme 1.

Mechanistic investigations[2] revealed that the tocopherol cyclase is operating by *si*-protonation of the double bond of **2** and concomitant *re*-attack of the phenolic O-atom, Scheme 1. Subsequent purification of the enzyme proved to be very difficult because the protein is membrane bound and extremely hydrophobic. Nevertheless, it was possible to prepare a stable $(NH_4)_2SO_4$ precipitate and an acetone powder; the latter was freeze-dried and extracted with organic solvents to remove the terpenes completely. The tocopherol cyclase was subsequently dissolved in the presence of the non-ionic detergent lauryl maltoside. After several chromatographic steps including Mono-Q, Mono-P, and size exclusion chromatography the 42 kD protein was obtained pure by SDS gel electrophoresis (purification factor PF: 13.850).[3] In order to possibly improve the purification procedure in the presence of a substrate analogue, and furthermore to understand the recognition of the substrate by the protein's active site, we investigated the substrate specificity of the enzyme both in its enriched form and as intact spheroplasts of *Anabaena variabilis*.

Results and Discussion

The enzyme tocopherol cyclase plays a key role in the biosynthesis of the chromanol substructure of the vitamin E family,[1] and it was uncertain, before we identified this protein, whether the cyclase would act on the hydroquinone **2** or the corresponding quinone.[4] It was also believed that α-tocopherol **3**, the biologically most active member of the vitamin E compounds, is exclusively produced in nature by methylation of γ-tocopherol **1**. Thus we first investigated substrate analogues of **2** modified at the periphery of the aromatic ring (Scheme 2).

Incubations of compounds **4–9** under standard conditions[1,2] with hydrophobic interaction chromatography (HIC)-purified enzyme fractions and with tocopherol cyclase twice purified by chromatography on Mono-Q revealed that the precursors **5–9** were cyclized (yields

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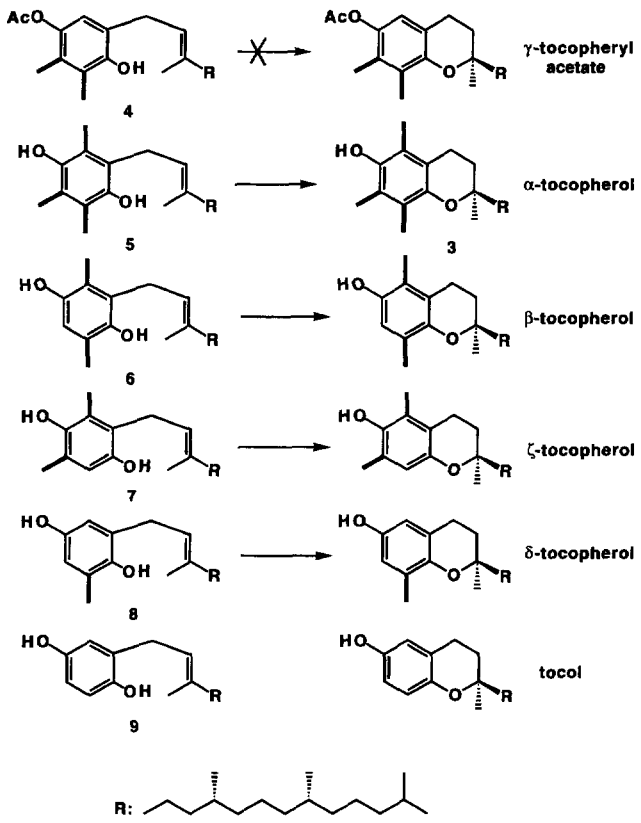
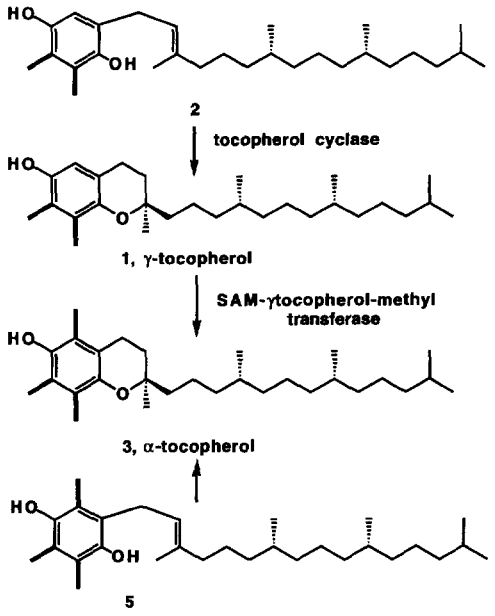


Scheme 2.

18–70%) to the corresponding chromanols irrespective of their degree and position of methylation at the aromatic ring. In contrast, **4** was not cyclized with either intact spheroplasts or with the purified enzyme. These results suggest that the OH group at C(1) of **2** is important for substrate recognition most likely by formation of a H-bridge to an amino acid of the substrate binding domain. It is interesting to note, however, that [2-methyl-^{3}H,^{14}C]-**5** in incubations with spheroplast, and unlabelled **5** when incubated with the purified enzyme yielded α-tocopherol **3** in 35% and 18% yield, respectively. In contrast to earlier reports,[4] these results suggest two possible alternative routes for the biosynthesis of **3**: (i) direct cyclization of the trimethylated precursor **5**, and (ii) methylation of **1** by γ-tocopherol-methyltransferase (Scheme 3). The latter enzyme has been identified in other organisms by Camara et al.,[5] and we have confirmed its existence in *Anabaena variabilis* by experiments with spheroplasts that gave both **1** and **3** from (radioactively labeled) **2** under prolonged incubation conditions.

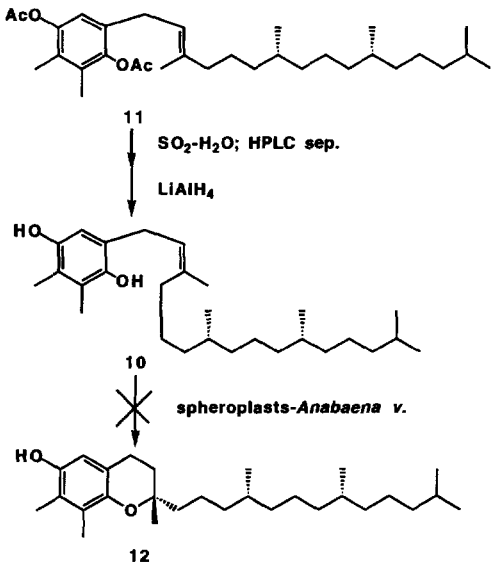
The significance of the configuration of the double bond of **2** was investigated by means of **10** that was prepared according to standard procedures by heating the diacetate **11** in SO_2/H_2O at 50 °C in a sealed tube.[6] The resulting *E/Z* mixture was separated by HPLC, and the (*Z*)-diacetate reduced to **10**. After incubation of **10** with *Anabaena* spheroplasts under standard conditions no traces of the γ-tocopherol epimer **12** could be detected (Scheme 4). Obviously the *E* double



Scheme 3.

bond of the precursor (e.g., in **2**) is a further recognition site for the tocopherol cyclase.

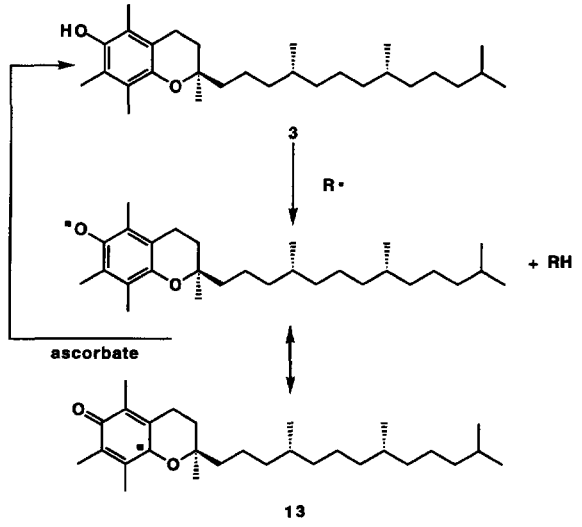
The chemical reactivity of the tocopherols is related to the chromanol part due to its capability to donate H· to radicals produced by oxidative processes, and stabilize the resulting radical **13** in the quinoid system (Scheme 5). However, the hydrophobic side chain of the tocopherols is equally important because it determines the site of action, and hence it is nowadays accepted that these compounds act in membranes as the most important radical chain-breaking antioxidants.[7] Accordingly, it is of general interest to determine the substrate specificity of tocopherol cyclase with respect to side chain modifications. The main questions were how specific is the enzyme concerning the



Scheme 4.

chirality in the phytyl substructure of **2**, and in particular concerning the length of the lipophilic chain.

Subsequently, we prepared a hydroquinone precursor **rac-2** with a racemic phytyl-chain. After incubation of **rac-2** under standard conditions the tocopherol extract was methylated and subjected to GLC analysis[1,8] revealing that the enzyme has produced a mixture consisting of (*R,R,R*)-**1** (48%), (*R,S,S*)-**1** (23%), (*R,R,S*)-**1** (16%), and (*R,S,R*)-**1** (13%) (Scheme 6). Obviously, the tocopherol cyclase has absolute preference to yield the *R*-configuration at C(2), moreover, the product with natural (*R,R,R*) configuration is formed about four-times faster than (*R,S,R*)-**1**. If one attributes this kinetic resolution to binding forces of the respective precursors there is seemingly very little discrimination due to the chiral centers in the phytyl-part, in fact less than 1 kcal difference of binding energy.

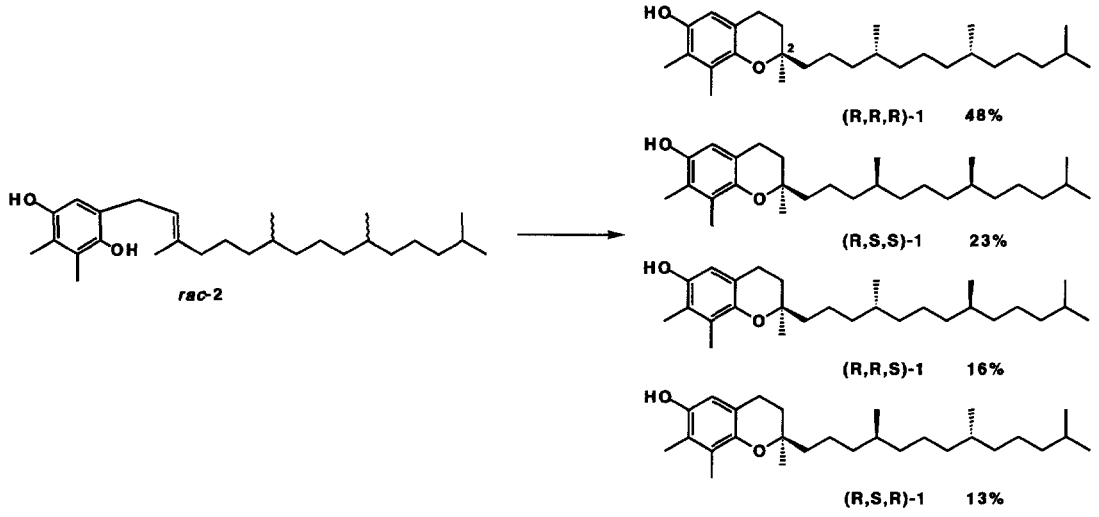


Scheme 5.

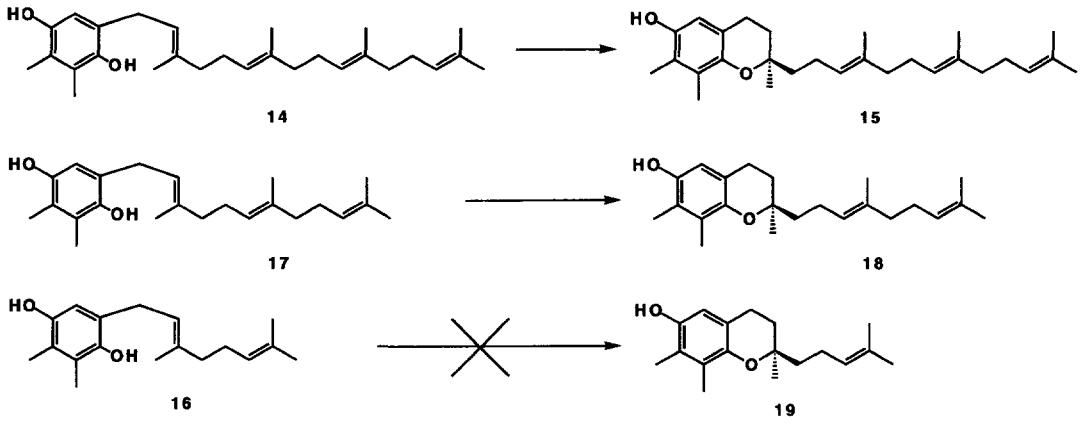
To address the second question concerning the significance of the length of the side chain's substrate for recognition we took advantage of an experiment with the tocotrienol precursor **14**, which was cleanly converted to γ-tocotrienol **15** in 83% yield both by spheroplasts and the purified enzyme (Scheme 7). The tocotrienols often accompany the vitamin E group in different tissues, however not in *Anabaena*. The tocotrienols are less potent antioxidants than the tocopherols, however they have been recently shown to act as inhibitors of the biosynthesis of cholesterol,[9] apparently by down-regulation of the rate-limiting enzyme HMG-CoA reductase. It is interesting to note that in our experiment no γ-tocopherol **1** could be detected, indicating that reduction of the side chain's double bond is only occurring at an earlier stage of the biosynthesis, that is, either phytyl-PP or geranylgeranyl-PP are condensed with homogentisinic acid to yield different hydroquinone precursors **2** and **14** which are cyclized by the same enzyme.

The advantage of the tocotrienol experiment is that compounds having shorter chains are much easier to synthesize than the corresponding chiral nor-tocopherols. The geranyl- and farnesyl-hydroquinones **16** and **17** were prepared from the allylic bromides by condensation with the methoxymethyl (MOM)-protected, lithiated hydroquinones and subsequent acidic cleavage and reduction, see Scheme 9 and corresponding experiments. Incubations with tocopherol cyclase revealed that **16** is no substrate, whereas **17** is cyclized to **18** in 14% yield (Scheme 7). Accordingly the hydrophobic interaction between the substrate's tail and the hydrophobic pocket of the enzyme is extremely important for recognition.

In view of these results, three main recognition sites for the hydroquinone precursor can be identified (see Scheme 8) however it was uncertain whether the substrate approaches the enzyme's active site *head-first* or *tail-first*. In order to investigate this problem and



Scheme 6.

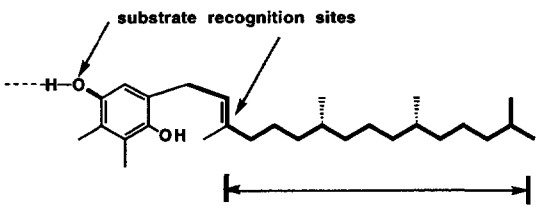


Scheme 7.

coincidently improve the purification of the protein, we decided to prepare the affinity matrices **20** and **21** which are distinct with respect to their attachment to the solid support, see Scheme 9.

Compound **20** was prepared according to Azzi et al.,[10] whereas the synthesis of **21** was pursued starting with geranylgeraniol **22**. Conversion of **22** to the allylic bromide **23**[11] was followed by the coupling with the lithiated compound **24** in the presence of CuBr to yield the MOM-protected hydroquinone **25**. Functionalization at its dimethyl allyl terminus by oxidation with SeO_2 and *tert*-butyl hydroperoxide (TBHP) produced the allylic alcohol[12] which then was esterified[13] with the *N*-allyloxycarbonyl protected β-alanine **26**.[14,15] Cleavage of the methoxymethyl ether with CAN $(Ce(NH_4)_2(NO_2)_6)$[16] and concomitant oxidation of the liberated hydroquinone to the benzoquinone was followed by the Pd^0 catalysed removal of the *N*-allyloxycarbonyl group yielding the amine **27**.[15] Finally, the coupling of **27** to *N*-hydroxysuccinimide ester activated sepharose gel and the reduction of the benzochinone to the hydrochinone with ascorbate furnished the affinity gel suitable for binding studies with tocopherol cyclase.

When the enzyme was loaded on a small column containing **21**, the total cyclase activity was found in the flow-through. In contrast, **20** retained the enzyme activity on the column and also behaved as a competitive inhibitor of the reaction **2** to **1**, as shown in a standard incubation experiment. These results suggest that the substrates of tocopherol cyclase enter the active site of the enzyme with the hydrophobic tail.



Scheme 8.

Experimental

General[1,2]

Tocopherols, tocotrienols, and their respective hydroquinone precursors were obtained from F. Hoffmann-LaRoche AG.

Incubations

Incubations of the hydroquinone precursors were carried out under standard conditions with *Anabaena* spheroplasts,[1] solubilized acetone powder,[2] and tocopherol cyclase purified by HIC, and anion exchange chromatography (Mono-Q/Mono-P).[3] The chromanols were isolated, and their yields determined by GLC/HPLC[1,2] by comparison with authentic samples.

(*E,E,E*)-1-Bromo-3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraene (23). According to ref. 11, Me_2S (0.558 g, 9.0 mmol) was added dropwise to a vigorously stirred cold (0 °C) solution containing NBS (1.35 g, 7.5 mmol) in anhydrous CH_2Cl_2 (25 mL). The yellow reaction mixture was cooled to −20 °C, and a solution of geranylgeraniol (**22**, 1.45 g, 5.0 mmol) in CH_2Cl_2 (5 mL) was added dropwise over a few minutes. After stirring for 3 h at 0 °C (reaction control by TLC, toluene:EtOAc, 4:1) the reaction mixture was diluted with hexane and poured into ice water. The organic layer was separated and washed with cold brine, dried ($MgSO_4$), filtered, and concentrated to give 1.65 g of **23** as a white solid (93.8%), CI-MS: 354.4 $[M+H]^+$.

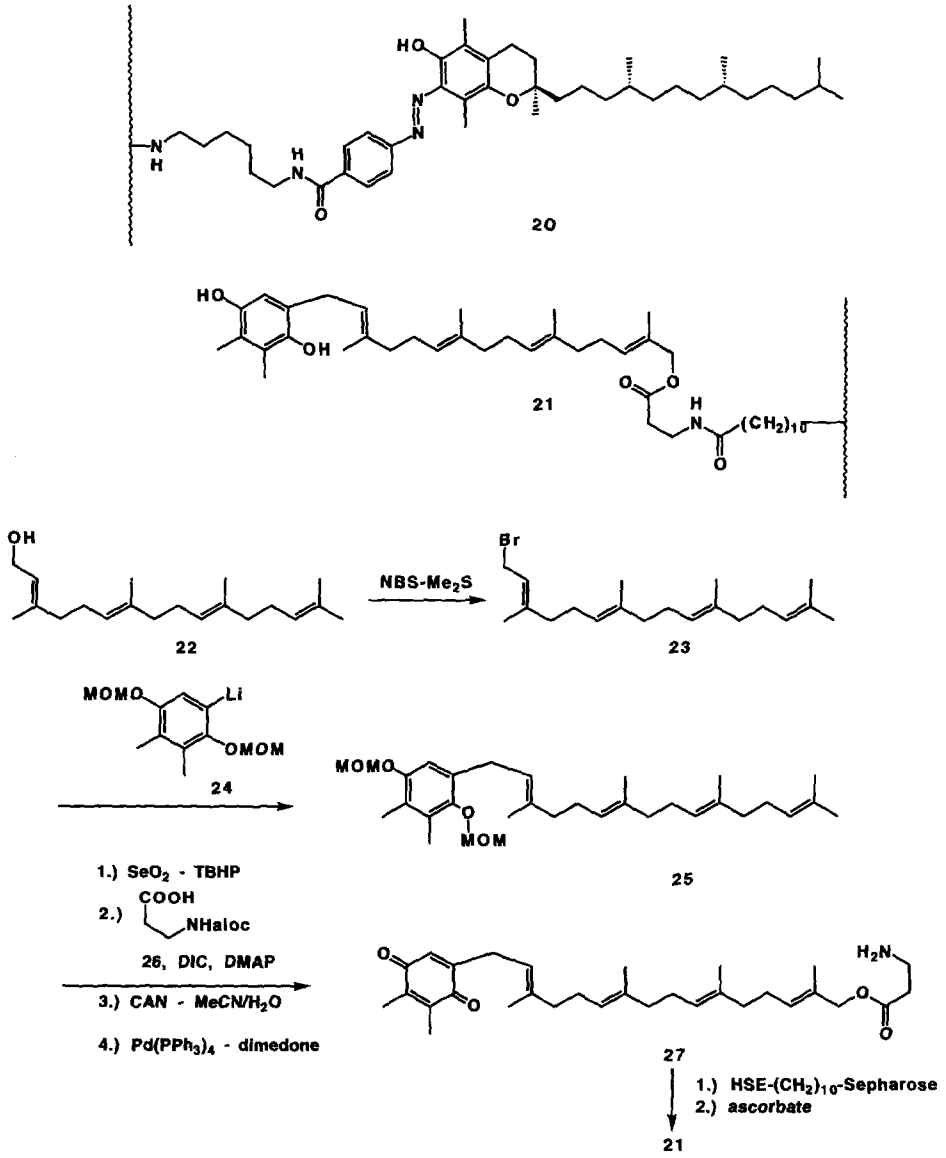
(*E,E,E*)-2,3-Dimethyl-5-(3′,7′,11′,15′-tetramethyl-2′,6′,10′,14′-hexadecatetraenyl)-1,4-hydroquinone-bis-methoxymethyl ether (25). A solution of BuLi in hexane (5.9 mL, 0.7 M, 4.13 mmol) and TMEDA (0.48 g, 4.1 mmol) were added to THF (25 mL). After cooling to 0 °C a solution of **24** (0.93 g, 4.1 mmol) in THF (5 mL) was added dropwise and the reaction mixture stirred for 2 h at 0 °C before adding CuBr (59 mg, 0.4 mmol). At −20 °C a solution of **23** (1.61 g, 4.55 mmol) in THF (7 mL) was added dropwise to the grey mixture. After stirring for 30 min at −20 °C and for 2 h at room temperature the reaction was

quenched with acetone (1 mL). Dilution with hexane (50 mL), followed by filtration on Celite, and evaporation of the solvent, gave 2.1 g of a brownish oil from which 1.37 g (66.8%) of the desired product, compound **25**, could be isolated as a colorless oil by flash-chromatography (toluene:hexene, 1:1), CI-MS: 499.8 $[M+H]^+$.

***(E, E, E, E)*-2,3-Dimethyl-5-(16′-aminoethylcarbonyloxy-3′,7′,11′,15′-tetramethyl-2′,6′,10′,14′-hexadecatetraenyl)-1,4-benzoquinone (27).** To a magnetically stirred suspension of SeO_2 (24.2 mg, 0.22 mmol in CH_2Cl_2 (0.35 mL) a solution of TBHP (293 mL, 3 M in iso-octane, 0.88 mmol) was added and stirred for 30 min at ambient temperature. Subsequent addition of a solution of **25** (219.5 mg, 0.44 mmol) in CH_2Cl_2 (0.25 mL) gave a clear-yellow solution that was stirred for further 30 min. After evaporation of the volatiles, the residue was dissolved in Et_2O and the solution washed with 10% aqueous Na_2CO_3 and brine. Separation of the products by flash-chromatography (hexane:EtOAc) gave 21.0 mg aldehyde and 19.5 mg (8.6%) of desired allylic alcohol. CI-MS: 515.9 $[M+H]^+$.

Diisopropyl carbodiimide (5.0 mg, 0.04 mmol) was added to a stirred solution of the allyl alcohol (18.0 mg, 0.035 mmol), followed by Aloc-β-AlaOH (**26**, 6.3 mg, 0.036 mmol) and DMAP (0.5 mg) in CH_2Cl_2 (0.25 mL) at −10 °C. After stirring for 1 h at −10 °C the reaction mixture was warmed up to room temperature and was stirred for further 12 h. The solvent was removed in vacuo and the product was isolated by flash-chromatography (hexane:EtOAc, 4:1) giving 20.6 mg (88.0%) of the *N*-aloc-protected compound, CI-MS: 670.9 $[M+H]^+$.

In order to remove the MOM groups and concomitant oxidation to the benzoquinone moiety, an aqueous solution (0.15 mL H_2O) of $Ce(NH_4)_2(NO_2)_6$ (39.0 mg,



Scheme 9.

0.071 mmol) was added to a cooled solution (0 °C) of the *N*-aloc-protected compound (20.0 mg, 0.03 mmol) in MeCN (0.15 mL) and the reaction mixture was kept stirring at the same temperature for 30 min. The mixture was poured onto satd aqueous $NaHCO_3$. After extraction with EtOAc, the combined organic layers were washed with brine, dried with $MgSO_4$, filtered, and the solvents evaporated to give 16.3 mg (93.7%) of a dark-yellow oil. CI-MS: 580.8 $[M+H]^+$.

This compound (15.6 mg, 0.027 mmol) was treated without further purification in a degassed solution of THF with $Pd(PPH_3)_4$ (12.4 mg, 0.01 mmol) in the presence of dimedone (30.3 mg, 0.216 mmol). After stirring for 2 h at 30 °C, the reaction was complete as shown by TLC. The solvent was removed in vacuo and the residue dissolved in Et_2O. After removal of a precipitate by filtration, the yellow solution was washed with 5% aqueous Na_2CO_3, H_2O, brine, dried with $MgSO_4$, and the solvent evaporated to give **27** as a dark-yellow oil, CI-MS: 496.8 $[M+H]^+$.

Anhydrous coupling of 27 to Affi-Gel®10 (Bio-Rad). The amine **27** (10 mg, 0.02 mmol) was dissolved in 1 mL of dioxane and the solution added to a suspension of *N*-hydroxysuccinimide-activated Affi-Gel® 10 (3.5 mL, 0.015 mmol/mL), pre-equilibrated in dioxane (3.5 mL). Finally, 0.1 mL Hünig's base was added and the suspension was smoothly shaken at room temperature for 2 h. No residual **27** could be detected in the supernatant by TLC. After the gel was washed with dioxane (5 × 4 mL) and cold H_2O (3 × 4 mL) phosphate buffer (4 mL, pH 7.8) containing ethanolamine HCl (0.4 mL, 1 M) was added and the suspension kept shaking for 1 h at 0 °C in order to block any remaining active esters. After the final washings with H_2O (5 × 4 mL) a yellow gel was obtained which by treatment with an aqueous solution of ascorbate turned colorless.

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