115. Identification of the Tocopherol-Cyclase in the Blue-Green Algae *Anabaena variabilis* KÜTZING (*Cyanobacteria*)

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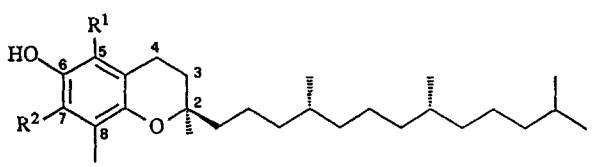
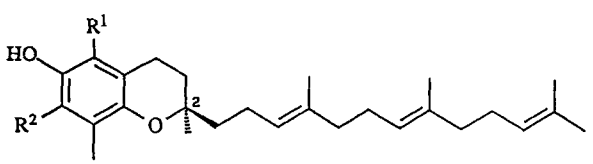
(1.IV.93)

Incubation of Spheroplasts of *Anabaena variabilis* (*Cyanobacteria*) with **22** the 2,6-*O*-dimethyl-β-cyclodextrin complex of 2,3-dimethyl-5-phytylhydroquinone (**11**), revealed the presence of a hitherto unknown enzyme which catalyzes the cyclization of the substrate **11** to give enantiomerically pure γ-tocopherol (**3**) in ≥ 93% yield.

Introduction. – The tocopherols **1–4** and the tocotrienols **5–8**, which can be isolated from photosynthetic organisms [1], are structurally related derivatives of chroman-6-ol displaying vitamin E activity [2]. The significance of these compounds for mammalian nutrition is known for almost 70 years [3]; however, our understanding of the basic principle of action of these compounds has only recently evolved. According to results obtained by *Burton* and *Ingold* [4] α-tocopherol **1**, the biologically most active member of the vitamin E group, is probably the most important lipophilic radical-chain-breaking anti-oxidans in living tissues. Common structural increments like the hydrophilic phenol and the lipophilic side chain at C(2) render these compounds easily incorporated into bilayer membranes [5] and, hence, protecting cell walls against autoxidative damage, in particular in the presence of ascorbic acid.



	R^1	R^2
1	CH_3	CH_3
2	CH_3	H
3	H	CH_3
4	H	H

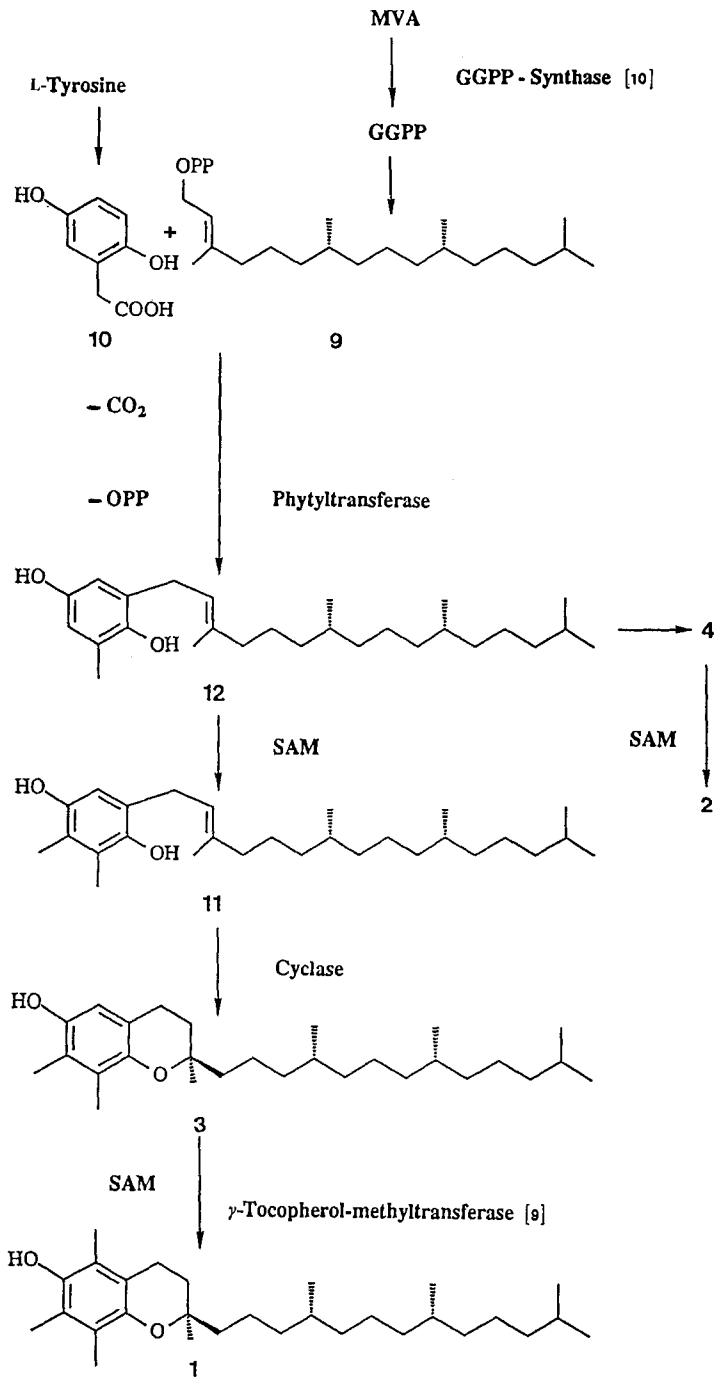


	R^1	R^2
5	CH_3	CH_3
6	CH_3	H
7	H	CH_3
8	H	H

In contrast to the tocopherols, the tocotrienols have been recently shown to act as inhibitors of the biosynthesis of cholesterol [6], appearently by down-regulation of the rate-limiting enzyme HMG-CoA reductase.

The biosynthesis of the tocopherols, tocotrienols, and side-chain homologues is well established involving the building blocks phytyl pyrophosphate **9** and homogentisinic

Scheme 1


MVA
GGPP - Synthase [10]
GGPP
L-Tyrosine
OPP
HO
OH
COOH
10
9
– CO_2
– OPP
Phytyltransferase
12
4
SAM
SAM
2
11
Cyclase
3
SAM
γ-Tocopherol-methyltransferase [9]
1

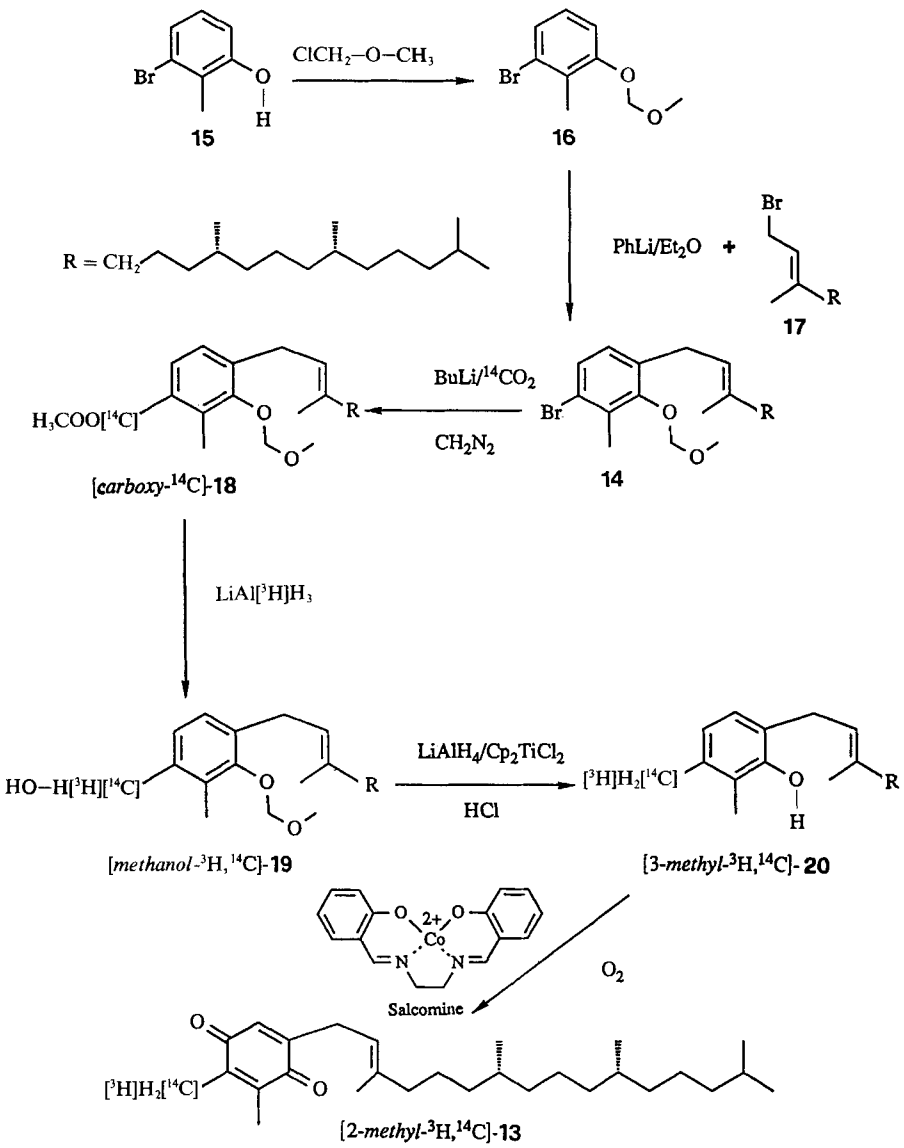
acid **10** (*Scheme 1*). The latter undergoes reductive decarboxylation with retention of configuration [7], and, therefore, is not only the source of the aromatic nuclei of **1**–**8** but also always supplies the Me group at C(8). All other Me groups originate from *S*-adenosylmethionine (SAM) at different stages of the pathway [8]. Mainly due the efforts of *Camara et al.*, several enzymes of this sequence have been isolated [9] [10]. However, in spite of many attemps in the last decade, it has not been possible to unumbiguously identify an enzyme catalyzing the cyclization of **11**→**3**. This situation and the challenge of possibly replacing a difficult reaction in the synthesis of (*R*,*R*,*R*)-tocopherols by an enzyme-mediated process led us to investigate the problem.

Results and Discussion. – Since most of the biosynthetic research by other laboratories [11] [12] was carried out with eucaryotic cells, or organelles thereof, we assumed that it would be of advantage using a procaryotic organism, in particular, since cloning of the enzyme was envisaged at a later stage of the work. Thus, the well known, autotrophic blue-green algae *Anabaena variabilis* KÜTZING (*Cyanobacteria*) was chosen. We were able to grow this organism and other members of the same genus [13] [14] according to the procedure of *Kratz* and *Myers* [15] in liquid cultures at 32° in the presence of light and a constant stream of air/2% CO_2 to yield *ca.* 3 g/l biomass after 7 days. To identify the tocopherols *ca.* 40 g of wet cells were harvested and freeze-dried to give *ca.* 4.5 g of a green powder. Cell disruption was completed by grinding under liquid N_2 and the remaining cell debris extracted for the tocopherols. After chromatographic separation from chlorophylls and carotenoids, a sample was obtained containing all four tocopherols **1**–**4** (95.8, 2.5, 0.4, 1.3%, respectively). To investigate the enzymatic formation of the chromane ring system of **3** from an acyclic precursor, the synthesis of the doubly-labelled quinone [2-*methyl*-^{3}H,^{14}C]-**13** was carried out according to *Scheme 2*. The ^{14}C-label was introduced by carboxylation of the Li salt derived from the bromo-ether **14**, which was accessible from the bromophenol **15** by condensation of the corresponding bromo-ether **16** with bromide **17**. The ^{3}H-label was attached to the ^{14}C-labelled C-atom on treatment of the ester [*carboxy*-^{14}C]-**18** with LiAl[^{3}H]H$_3$ to yield [*methanol*-^{3}H,^{14}C]-**19**. Further reduction followed by acid ether cleavage yielded [3-*methyl*-^{3}H,^{14}C]-**20** carrying the desired doubly-labelled methyl group, ^{3}H/^{14}C = 2.2; salcomine oxidation (bis(salicylidene) ethylenediiminocobalt(II)) finally gave the benzoquinone [2-*methyl*-^{3}H,^{14}C]-**13**.

When [2-*methyl*-^{3}H,^{14}C]-**13** was incubated in a suspension of intact cells of *Anabena variabilis* in phosphate buffer/*Tween80* (pH 7) for 15 h, no chromane ring formation could be detected by radioactive TLC scanning. However, if [2-*methyl*-^{3}H,^{14}C]-**13** was treated with $Na_2S_2O_4$ prior to incubation, a small amount (*ca.* 0.1% conversion) of labelled γ-tocopherol **3** and α-tocopherol **1** was observed (10:1). After dilution with nonradioactive reference compounds, the 4-bromobenzoates of **1** and **3** were prepared and recrystallized until constant specific radioactivity; the ^{3}H/^{14}C = 2.2 of these samples was found to be identical with the ^{3}H/^{14}C of the precursor [2-*methyl*-^{3}H,^{14}C]-**11**. Thus, it seemed that not **13** but the corresponding hydroquinone **11** is the precursor of **3** and **1**. At this point, we reasoned three main problems concerning the transformation of **11**→**3**: *i*) low solubility of **11** in the buffer, *ii*) fast oxidation of **11**→**13** during incubation, and *iii*) slow transport of **11** through the cell wall of *Anabaena variabilis*.

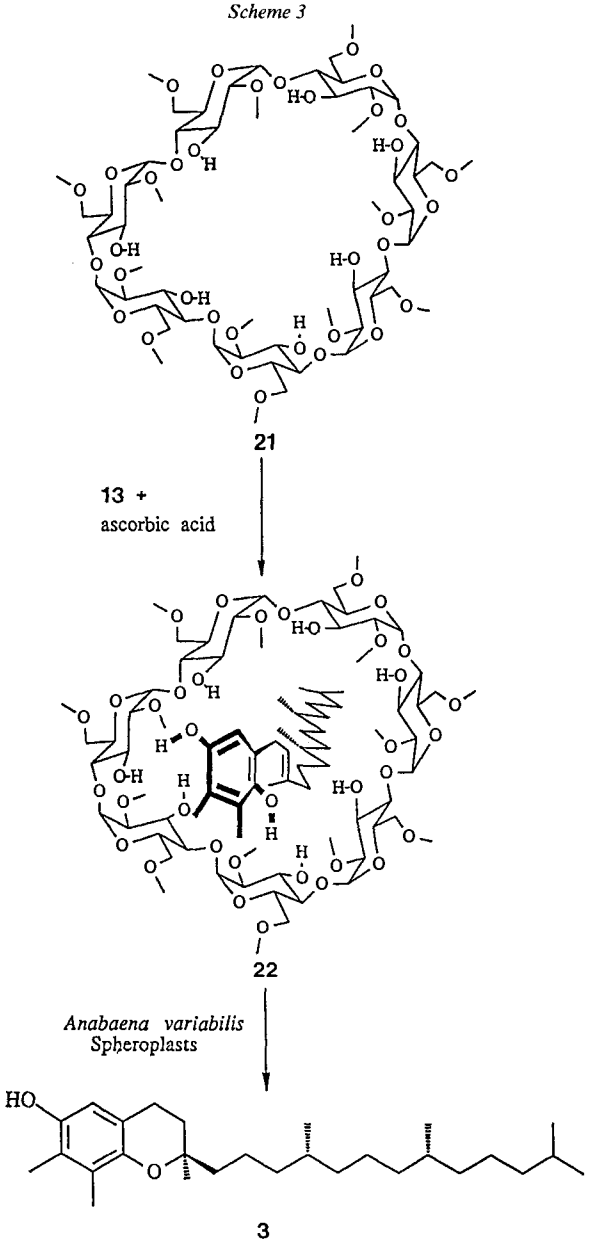
The low solubility of **11** (and **13**) in H_2O should be considerably enhanced by masking the hydrophobic side chain. In fact the addition of an excess of 2,6-di-*O*-methyl-β-

Scheme 2



cyclodextrin (**21**) [16] to a suspension of **13** in phosphate buffer/D_2O gave a clear yellow solution displaying a characteristic bathochrome UV change and in the ^{1}H-NMR spectrum downfield shifts for all protons of the Me groups at the side chain (0.08–0.16 ppm), most significantly for the vinylic Me group, indicating the side chain of **13** is buried in the cavity of the cyclodextrin (*Scheme 3*). The inclusion complex of **13** was easily reduced in the presence of ascorbic acid to yield a colorless solution of **22**, the cyclodextrin complex

Scheme 3



of **11**; the transformation was quantitative as shown by UV (260 nm (**13**)→298 nm (**11**)). As it turned out later, an excess of ascorbic acid serves perfectly suppressing the oxidation of **11** during the incubation.

To facilitate the transport of **11** to the site of biosynthetic activity, the outer glycosidic part of the cell wall was degraded by incubating *Anabaena variabilis* cells with lysozyme at

pH 7/35°. Control of the lysozyme fermentation by phase-contrast microscopy revealed that the long-chain filaments ($\geq$ 50 cells/string) of cylindrical *Anabaena* cells morphologically changed to shorter aggregates (5–10 cells/string) and individual cells of ball-shaped spheroplasts [17], a process which was completed up to 85% within 3 h. Osmolysis of the spheroplasts was suppressed by enhanced concentration of sucrose (500 mM) in the buffer. Incubation of the soluble cyclodextrin complex of [2-*methyl*-^{3}H,^{14}C]-**11** with *Anabaena* speroplasts in the presence of ascorbic acid for 18 h gave doubly labelled γ-tocopherol [7-*methyl*-^{3}H,^{14}C]-**3** in reproducible yields of 93% as shown by HPLC/radioactivity detection. As it turned out later, the conversion **11**→**3** was nearly quantitative indeed, since 50% of the uncyclized material was identified as the (*Z*)-isomer of [2-*methyl*-^{3}H,^{14}C]-**11** [18]. A control experiment revealed that no traces of **3** are produced from **22** in the absence of enzyme under otherwise identical conditions of incubation. Furthermore, it was shown by GLC [19] of [7-*methyl*-^{3}H,^{14}C]-**23**, the methyl ether of

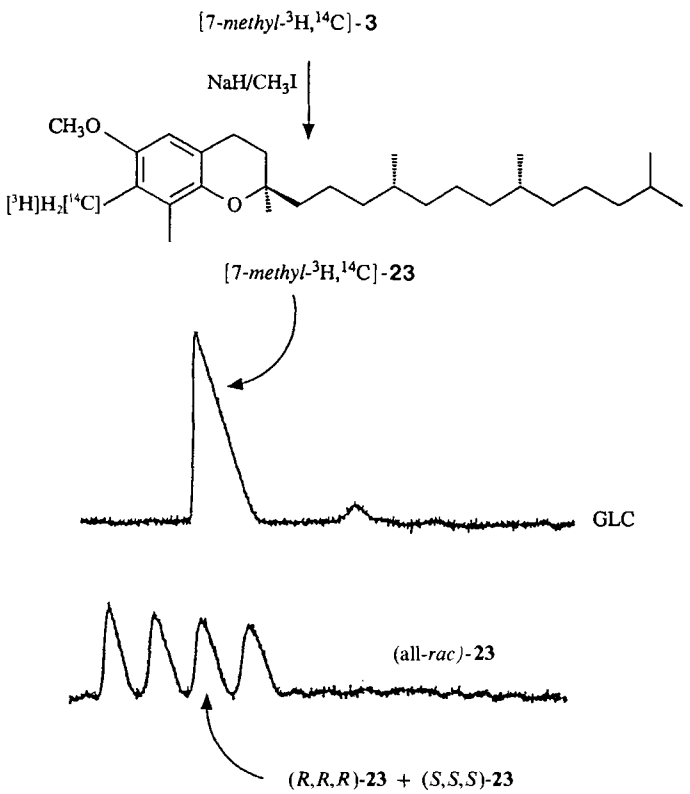


Figure. *GLC of* [7-methyl-$^3H,^{14}C$]-**23** *and all*-rac-**23**

[7-*methyl*-^{3}H,^{14}C]-**3**, that the γ-tocopherol obtained from incubation had the natural (*R*,*R*,*R*)-configuration (*Fig.*).

Thus, for the first time an enzyme has been identified in the blue green algae *Anabaena variabilis*, which catalyzes the diastereospecific cyclization of the hydroquinone precursor

11 to γ-tocopherol **3**. Further work is in progress to isolate the *tocopherol-cyclase* [20], to investigate its substrate specificity [18], and the mechanism of ring closure [21].

We are indepted to the following colleagues at *F. Hoffmann-La Roche AG* (Basel) for their invaluable help in the course of this investigation: Dr. *L. Chopard* (IR spectra), Dr. *W. Arnold* (NMR spectra), Dr. *W. Vetter* and *W. Meister* (mass spectra), Dr. *A. Dirscherl* (elemental analysis), Dr. *M. Vecchi, W. Walther*, and *E. Glinz* (GC, HPLC); Dr. *J. Würsch* and *P. Dreier* (synthesis of doubly labelled **13**), *M. Kaeggli* and *P. Loranz* (synthesis of **13**). *A.S.* and *W.-D. W.* thank *F. Hoffmann-La Roche AG* for generous financial support of their work in Zurich. Finally, we thank our colleagues in Basel and Zürich for stimulating discussions in particular Dr. *R.K. Müller*, Dr. *H.J. Mayer*, and Prof. *H.-J. Hansen*.

Experimental Part

General. If not otherwise stated, all reagents and solvents were of *puriss.* quality and purchased from *Fluka AG*, Buchs, or *Merck AG* and were used without further purification. All reactions were carried out under Ar. THF and Et_2O were distilled from $LiAlH_4$. $CHCl_3$ was filtrated through Al_2O_3 (*ICN Biomedicals*). Hexane was distilled from CaH_2. H_2O was purified in a *Mili-Q* filtration unit, *Millipore*. An *IEC Centra-7R* centrifuge was used for prep. centrifugations. The radioactivity of the labelled compounds was determined by measuring aliquots in a *Packard Tri-Carb* liquid scintillation analyzer, model *2000 CA*, window ^3H: 0–12 eV, window ^{14}C: 12–156 eV; the measured events, cpm, were transformed to absolute decays, dpm, by computing with the aid of a quenchcurve. TLC: on silica-gel plates *Merck*, SiO_2 *60* F_{254}. Labelled compounds were detected by a *Berthold II LB 2723* TLC scanner, ^{14}C window. Column flash chromatography, (FC), on silica gel, 0.040–0.063 mm, 230–400 mesh, *Merck*. HPLC: *Lichrosorb SI60*, 5 µm. Labelled compounds were detected by a *Berthold LB 506C* HPLC scanner, separate ^{14}C and ^3H windows. Unlabelled compounds were detected by a *Waters Lambda-Max 481* UV/VIS spectrometer or by a *Merck F 1050* fluorescense spectrometer. GLC: cap. column *PS-086*, 18 m; capillary column *SE-54*, 25 m. UV Spectra: *Uvicon 810/820* spectrometer, *Kontron*; *Hewlett Packard 8452 A Diode Array* spectrometer, λ_{max} in nm (log ε). IR Spectra: *Nicolet 7199 FT-IR* spectrometer. H-NMR Spectra: *Bruker Spectrospin WM-250*, *Bruker AC-300*, *Bruker AM-400*; $CDCl_3$ solns.: if not otherwise stated, δ in ppm relative to TMS, coupling constants J in Hz. MS: *MS9 AEI*, Manchester, GB, *Varian MAT 112S*, *Finnigan 90* spectrometer; indication of characteristic peaks in m/z (% rel. to base peak (= 100%)).

Medium C (cultivation of *A. variabilis*): 1 l dest. H_2O containing: $MgSO_4 \cdot 7\,H_2O$ (0.150 g), K_2HPO_4 (0.600 g), $Ca(NO_3)_2 \cdot 4\,H_2O$ (0.010 g), KNO_3 (0.500 g), Na-citrate $\cdot 2\,H_2O$ (0.165 g), $Fe_2(SO_4)_3 \cdot 6\,H_2O$ (0.004 g), 1 ml *Solution TE* (trace elements). *Solution TE:* 1 l dest. H_2O containing H_3BO_3 (2.860 g), $MnCl_2 \cdot 4\,H_2O$ (1.810 g), $ZnSO_4 \cdot 7\,H_2O$ (0.222 g), MoO_3 (0.015 g), $CuSO_4 \cdot 5\,H_2O$ (0.079 g). Hypertonic *Buffer HB* (production of spheroplasts): sucrose (500 mM), phosphate buffer (30 mM; pH 7), $MgSO_4 \cdot 7\,H_2O$ (4 mM), EDTA $\cdot$ 2 Na (0.175 mM). Substrate *Buffer S* (incubation of various substrates with spheroplasts): hypertonic *Buffer HB*, 2.40 mM precursor, 45.1 mM 2,6-di-*O*-methyl-β-cyclodextrin (**21**), 250 mM ascorbic acid.

Conditions for Cultivating Anabaena variabilis KÜTZING. An agar slant culture of *A. variabilis* SAG 1403-4b was inoculated in 100 ml of *Medium C* in a 200-ml flask and cultivated at 32° for 6 d while bubbling *Medium C* with a mixture of air/CO_2 (2% CO_2) under permanent illumination with 6 bulbs (*Philips TLD* 15 W/33). After sedimentation for 10 min, the overstanding medium was decanted and the residual culture was inoculated in 1.8 l of *Medium C* in a 2-l flask. Cultivation was carried out under the above mentioned conditions for 6 d. After decantation, the residual culture was inoculated in 22 l of *Medium C* in two 12-l flasks and grown as described above. After 7 d, the cells were collected by centrifugation at 1000 *g* to yield a pellet at a net weight of 3–3.5 g/l of *Medium C*.

Determination of Tocopherols in Anabaena variabilis. Wet cells (40 g) of *A. variabilis* were lyophylized overnight to yield 4.5 g of dried cells, which were ground in a mortar under liquid N_2. The resulting powder was packed into a column containing 40 g of *CF11* Cellulose (*Whatman*). After addition of 100 ml of MeOH, the mixture was stirred with a glasrod to yield a dark green suspension. Elution of the MeOH phase was achieved by applying 0.5 bar N_2 on top of the column. The residue was treated twice as described above with a mixture of MeOH/$CHCl_3$ 2:1, until no further chlorophyll could be eluted. The combined eluates were evaporated yielding a crude extract (490 mg). Chromatography on a mixture of 40 g of silica gel and 20 g *Celite* (petroleum ether/Et_2O

8:2) afforded a yellow fraction (48 mg) containing the tocopherols. FC on 100 g of silica gel (hexane/i-PrOH 99:1) yielded three tocopherol-containing fractions which were combined and quantitatively identified by GLC and co-injection of authentic samples of known concentration. The mixture contained *α-tocopherol* (**1**); 193.1 μg/g, 95.8%), *β-tocopherol* (**2**; 5.0 μg/g, 2.5%), *γ-tocopherol* (**3**; 0.8 μg/g, 0.4%), and *δ-tocopherol* (**4**; 2.6 μg/g, 1.3%). The reproduction of this procedure is documented by the following figures obtained from an experiment with 3.7 g of dried cells of *A. variabilis:* **1** (233.8 μg/g, 96.3%), **2** (5.4 μg/g, 2.2%), **3** (0.9 μg/g, 0.4%), and **4** (2.7 μg/g, 1.1%).

Preparation of Spheroplasts. Wet cells (60 g) of *A. variabilis* were suspended twice in 400 ml of *Buffer HB* (1-l flask) followed by centrifugation (1500 *g*/5 min). The cells were then suspended in 800 ml of *Buffer HB* and converted to spheroplasts by adding lysozyme (800 mg, 78000 U/mg) and slowly rotating the flask at 35° for 3 h. The conversion to spheroplasts was monitored by phase-contrast microscopy every 30 min. The spheroplasts were collected by centrifugation at 4° (1500 *g*/10 min) and washed twice with 400 ml *Buffer HB* at 4° to yield a pellet of 52 g.

Preparation of the Cyclodextrin Complexes of **13** *and* **11**. *2,6-Di-O-methyl-β-cyclodextrin* (**21**, 0.15 mg, 113 μmol) was dissolved im 1.2 ml of D_2O. ^{1}H-NMR (400 MHz): 5.28–5.25 (*m*, 1 H–C(1)); 4.03–3.38 (*m*, 13 H); 3.60 (*s*, CH_3O–C(2)); 3.42 (*s*, CH_3O–C(6)). To the soln. of **21**, the benzoquinone **13** (10 mg, 24 μmol) was added (molar ratio 4.7:1; as confirmed by ^{1}H-NMR) to yield a slightly yellow soln. of the inclusion complex; ^{1}H-NMR (400 MHz): *a*) signals of the host: 5.20 (*s*, 1 H–C(1)); 4.03–3.38 (*m*, 13 H); 3.60 (*s*, CH_3O–C(2)); 3.40 (*s*, CH_3O–C(6)); *b*) signals of the guest: 6.54 (*s*, 1 H–C(6)); 2.09–2.04 (*m*, 8 H); 1.77 (*s*, CH_3–C(3′)); 1.61–1.00 (*m*, 19 H); 1.00–0.91 (*m*, 12 H). UV/VIS of **13** (32.6 μg, 78.6 nmol) in hexane (2 ml): 254 (19.00), 260 (17.60). Hexane was removed under a constant stream of N_2 and the residue dissolved in 2.0 ml of H_2O/22.54 mM cyclodextrin (**21**). After 5 min gently shaking the probe cuvette, the conversion to the cyclodextrin complex of **13** was complete; UV/VIS (H_2O): 260 (16.40); the reference cuvette contained 22.45 mM **21** in H_2O. Reduction of the cyclodextrin complex **13** was achieved by adding 18.2 mg (0.10 μmol) of ascorbic acid. After 3 h at r.t., the conversion to **22**, the cyclodextrin complex of **11**, was complete; UV/VIS (H_2O): 298 (3.02); the reference cuvette contained 2.0 ml of H_2O, 18.2 μg (0.10 μmol) of ascorbic acid, and the cyclodextrin (**21**; 22.45 mM).

Preparation of Substrate Buffer S. 4 ml of *Hypertonic Buffer HB* (4 ml) was transfered under Ar to a 25-ml flask containing **21** (600 mg, 0.45 mmol) and HPLC-pure [2-*methyl*-^{3}H,^{14}C]-**13** (10.0 mg, 24.0 μmol; ^{3}H = 37.1 mCi/mmol, ^{14}C = 16.5 mCi/mmol). After cooling the flask to 4° and gently rotating for 5 min, ascorbic acid (440 mg, 2.5 mmol) and *Buffer HB* were added to give 10 ml of a yellow soln. The flask was left at r.t. for 3 h and 1 h at 4°. The conversion of [2-*methyl*-^{3}H,^{14}C]-**13** to [2-*methyl*-^{3}H,^{14}C]-**11** was determined by extracting 0.2 ml of the substrate *Buffer S* with 0.2 ml of hexane in an 1-ml vial (*Eppendorf*). HPLC (combined with UV_{292} radioactivity detector) of 20 μl of the org. layer revealed the presence of ⩾ 97% pure [2-*methyl*-^{3}H,^{14}C]-**11** (t_R 8.1 min) and no detectable traces of [2-*methyl*-^{3}H,^{14}C]-**13** (t_R 2.9 min). The amount of substrate dissolved in *Buffer S* was confirmed by scintillation counting; [^{3}H,^{14}C]-yield ⩾ 98%. *Buffer S* was stable at r.t. for at least 48 h and could be stored at −20°.

*Conditions of Incubation of [2-*methyl-^{3}*H,*^{14}*C]-***11** *with* A. variabilis *Spheroplasts.* Spheroplasts (15 g) were suspended under Ar in a 100-ml flask in 50 ml of *Buffer HB* containing ascorbic acid (79.0 mg, 0.45 mmol). The enzymatic reaction was started by adding *Buffer S*: a soln. of [2-*methyl*-^{3}H,^{14}C]-**11** (0.20 mg, 0.48 μmol), **21** (12 mg, 9.0 μmol), and ascorbic acid (8.8 mg, 50.0 μmol) in 0.2 ml of *Buffer HB*. After sealing the flask under Ar, the suspension was incubated at 35°/120 rpm in a *Haake W19* shaker for 15 h. The enzymatic reaction was quenched by adding 150 ml of MeOH and the mixture extracted four times with 200 ml of hexane. After evaporating the combined hexane layers, the dark green residue was dissolved in Et_2O (10 ml), filtered through Na_2SO_4, and analyzed for radioactivity: recovered [^{3}H,^{14}C]$_{total}$: 96.3%. Radioactive TLC-scanning revealed one labelled compound having the same R_f as authentic γ-tocopherol (**3**). Final identification of [7-*methyl*-^{3}H,^{14}C]-**3** was accomplished on HPLC (combined with UV_{292}-radioactivity detector) by coinjection of unlabelled **3** (t_R 5.7 min). The [^{3}H,^{14}C]-recovery of [7-*methyl*-^{3}H,^{14}C]-**3** was determined as 93.1% (0.186 mg).

In a control experiment using spheroplasts denaturated prior by heat in *Buffer HB*, no labelled **3** could be isolated under otherwise identical incubation conditions.

*Absolute Configuration of [7-*methyl-^{3}*H,*^{14}*C]-***3**. By upscaling the above described incubation conditions, we were able to collect larger quantities (0.92 mg, 2.2 μmol, based on scint. count.) of [7-*methyl*-^{3}H,^{14}C]-**3**, which were first passed through a mixture of silica gel/*Celite* followed by FC on silica gel. Further purification was achieved by anal. TLC (petroleum ether/(i-Pr)$_2$O 7:3) followed by anal. TLC/$CHCl_3$ to furnish [7-*methyl*-^{3}H,^{14}C]-**3** (0.56 mg, 1.34 μmol, 56%, based on scint. count.). The compound was 96% GLC-pure and 100% pure on HPLC (combined with UV_{292} radioactivity detector). [7-*methyl*-^{3}H,^{14}C]-**3** was converted quantitatively to the corresponding methyl ether [7-*methyl*-^{3}H,^{14}C]-**23** on treatment with an excess NaH/CH_3I in abs. THF. The absolute configuration of the latter compound was determined on a chiral GLC column (*SP 2340*, 93 m) which separates synthetic (all-*rac*)-γ-tocopherol-methyl ether (all-*rac*)-**23** into four peaks corresponding to the four diastereoisomers. It was shown

that [7-*methyl*-^{3}H,^{14}C]-**23** has a retention time identical with (*R,R,R*)- and (*S,S,S*)-**23** and hence the natural (*R*)-configuration at C(2).

1-Bromo-3-(methoxymethoxy)-2-methylbenzene (**16**). To a cold (0°) soln. of *3-bromo-2-methylphenol* (**15**; 3.74 g, 20 mmol, m.p. 98.5°; [22]: 91–94°, [23]: 94.5°) and (i-Pr)$_2$NEt (7.75 g, 10.2 ml, 60 mmol) in CH_2Cl_2 (20 ml) was added dropwise *ca.* 6.1M $ClCH_2OMe$ (6.6 ml, 40 mmol, in AcOMe; *in situ* prepared according to [24]) within 30 min. After 1 h stirring at r.t., 25% aq. NH_3 (3 ml) was added and stirring continued for 1 h. Then, the mixture was poured into hexane (100 ml) and washed twice with 2M NaOH (50 ml) and brine, dried (Na_2SO_4), filtered, and evaporated: 4.0 g of yellow oil. Bulb-to-bulb distillation at *ca.* 100°/0.1 mbar afforded pure **16** (3.50 g, 76%) as colorless oil. GC: purity 100%. IR (neat): 2950, 1590, 1570, 1465, 1250, 1160, 1143, 1081, 1010, 769. ^{1}H-NMR (250 MHz): 2.34 (*s*, CH_3–C(2)); 3.48 (*s*, CH_3O); 5.19 (*s*, CH_2O); 7.0 (*m*, H–C(5), H–C(6)); 7.20 (*dd*, *J* = 7, 3, H–C(4)). MS: 232, 230 (each 7, M^+), 171, 169 (each 2), 90 (3), 78 (4), 45 (100). Anal. calc. for $C_9H_{11}BrO_2$ (231.09): C 46.78, H 4.80, Br 34.58; found: C 46.88, H 4.88, Br 34.44.

1-Bromo-3-(methoxymethoxy)-2-methyl-4-phytylbenzene (**14**)[1]. To a soln. of **16** (2.65 g, 11.5 mmol) in dry Et_2O (5 ml) was added at r.t. 1.3M PhLi (10 ml, 13 mmol, in Et_2O; freshly prepared [25] and titrated according to [26]). After refluxing for 7 h [27], the suspension was cooled to −25° and CuBr (144 mg, 1 mmol) was added, followed by treatment with a soln. of *phytyl bromide* (**17**) (4.95 g, 13.7 mmol; prepared from phytol (obtained from *Eisai Co., Ltd.* 5–5, Koishikawa 5, chome Bunkyo-ku, Tokyo, 112 Japan (content: 87.8% by GLC), and purified by chromatography before use) according to [28]) in Et_2O (10 ml). After stirring overnight at r.t. MeOH (2 ml) and hexane (100 ml) were subsequently added. Filtration through *Celite* and evaporation gave 7.1 g of crude product. Chromatography on 400 g of silica gel (toluene/hexane 1:2) afforded pure **14** (2.80 g, 48%) as a slightly yellow oil. IR (neat)[2]): 2920, 1575, 1460, 1160, 1080, 975. ^{1}H-NMR (250 MHz): *ca.* 0.85 (*m*, CH_3–C(7'), CH_3–C(11'), CH_3–C(15'), CH_3(16')); *ca.* 1.0–1.6 (*m*, 19 aliph. H); 1.68 (*s*, CH_3–C(3')); 2.00 (*t*, *J* = 7.5, 2 H–C(4')); 2.37 (*s*, CH_3–C(2)); 3.34 (*d*, *J* = 7.5, 2 H–C(1')); 3.61 (*s*, CH_3O); 4.94 (*s*, OCH_2O); 5.25 (*t*, *J* = 7.5, H–C(2')); 6.95 (*d*, *J* = 8, H–C(5)); 7.26 (*d*, *J* = 8, H–C(4)). MS[2]): 510, 508 (each 4, M^+), 478, 476 (each 8), 253, 251 (each 20), 201, 199 (each 20), 95 (16), 81 (25), 57 (38), 45 (100), 43 (32). Anal. calc. for $C_{29}H_{49}BrO_2$ (509.61)[2]): C 68.35, H 9.69, Br 15.68; found: C 68.27, H 10.08, Br 15.27.

Methyl 3-(Methoxymethoxy)-2-methyl-4-phytylbenzoate (**18**). To a cold (0°) soln. of **14** (3.43 g, 6.7 mmol) in dry Et_2O (15 ml) was added tetramethylethylenediamine (1.2 ml, 8 mmol) followed by 1.6M BuLi (5.1 ml, in hexane). After stirring for 1 h, the mixture was cooled to −25° and gaseous CO_2 (dried with conc. H_2SO_4) was passed through the soln. for 5 min. Acidification with 2N H_2SO_4, extraction with hexane (150 ml) and washing with H_2O and brine gave, after usual workup, crude acid (3.05 g) which was esterified with *ca.* 0.3M CH_2N_2 [29] (25 ml, *ca.* 7.5 mmol, in Et_2O) to give crude **18** (2.63 g). Chromatography on 130 g of silica gel (hexane/AcOEt 19:1) afforded pure **18** (1.86 g, 57%) as a colorless oil. IR (neat)[2]): 2927, 1725, 1599, 1275, 1258, 1160, 1045, 969. ^{1}H-NMR (250 MHz)[2]): *ca.* 0.85 (*m*, CH_3–C(7'), CH_3–C(11'), CH_3–C(15'), CH_3(16')); 1.0–1.6 (*m*, 19 aliph. H); 1.69 (*s*, CH_3–C(3')); 2.01 (*t*, *J* = 7, 2 H–C(4')); 2.52 (*s*, CH_3–C(2)); 3.44 (*d*, *J* = 7, 2 H–C(1')); 3.62 (*s*, CH_3O); 3.87 (*s*, CH_3O); 4.95 (*s*, OCH_2O); 5.28 (*t*, *J* = 7, H–C(2')); 7.07 (*d*, *J* = 8, H–C(4)); 7.60 (*d*, *J* = 8, H–C(5)). MS[2]): 488 (8, M^+), 456 (30), 245 (26), 204 (28), 179 (48), 57 (40), 45 (100). Anal. calc. for $C_{31}H_{52}O_4$ (488.75)[2]): C 76.18, H 10.72; found: C 76.40, H 10.65.

2,3-Dimethyl-6-phytylphenol (**20**). To a cold (0°) soln. of **18** (1.86 g, 3.8 mmol) in dry Et_2O (15 ml) was added slowly $LiAlH_4$ (150 mg, 3.9 mmol). After stirring for 1 h, H_2O was added. Extraction with hexane (150 ml), washing with 2N H_2SO_4, H_2O and brine gave, after usual workup, chromatographically pure **19** (1.64 g, 93%), which was directly used in the next step.

To a soln. of **19** (1.64 g, *ca.* 3.5 mmol) in dry THF (15 ml) was added titanocene dichloride (120 mg, 0.5 mmol, *Fluka*) and $LiAlH_4$ (600 mg, 16 mmol) [30]. The mixture was refluxed for 1.5 h, then additional titanocene dichloride (120 mg, 0.5 mmol) was added, and refluxing was continued for 1 h. After cooling to r.t., the mixture was treated with H_2O and worked up as usual to give an oil (1.4 g) which was dissolved in THF/*i*-PrOH/AcOH/HCl (37%) 10:10:10:1 (*v/v*; 15 ml, freshly prepared) and stirred at r.t. for 4 h. Pouring into H_2O and extraction with hexane gave, after usual workup, crude **20** (1.13 g). Chromatography on 130 g of silica gel (toluene) yielded **20** (970 mg, 64%) as a colorless oil. ^{1}H-NMR (250 MHz): *ca.* 0.85 (*m*, CH_3–C(7'), CH_3–C(11'), CH_3–C(15'), CH_3–C(16')); 1.0–1.6 (*m*, 19 aliph. H); 1.79 (*s*, CH_3–C(')); 2.02 (*t*, *J* = 7, 2 H–C(4')); 2.15, 2.24 (2 *s*, CH_3–C(2), CH_3–C(3)); 3.35 (*d*, *J* = 7, 2 H–C(1')); 5.22 (*s*, OH); 5.30 (*t*, *J* = 7, H–C(2')); 6.68, 6.84 (2 *d*, *J* = 8, H–C(4), H–C(5)).

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[1]) Phytyl = *(2E,7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl.*

[2]) These data originate from another experiment carried out under similar conditions, which affords an identical product.

2,3-Dimethyl-5-phytylcyclohexa-2,5-diene-1,4-dione **13**. A soln. of **20** (970 mg, 2.4 mmol) and salcomine [31] (80 mg, 0.24 mmol) in EtOH (10 ml) was stirred in an O_2 atmosphere (1.2–1.4 atm.) for 1 h at *ca.* +10°. Evaporation of the solvent and chromatography on 130 g of silica gel (toluene) gave **13** (630 mg, 63%) as a golden oil. HPLC: 98.8% (2′*E* + 2′*Z*); (2′*E*/2′*Z*) ~ 98:2. UV/VIS (hexane): 309 (2.86), 261 (4.24), 253 (4.27); [11]: (petroleum ether): 260 (4.22), 254 (4.25). IR (neat): 2926, 2867, 1652, 1616, 1462, 1378, 1316, 1165, 1100, 882. ^{1}H-NMR (250 MHz): *ca.* 0.85 (*m*, CH_3–C(7′), CH_3–C(11′), CH_3–C(15′), CH_3–C(16′)); 1.0–1.6 (*m*, 19 aliph. H); 1.61 (*s*, CH_3–C(3′)); 2.01, 2.03 (2 *s*, CH_3–C(2), CH_3–C(3)); 3.12 (*d*, *J* = 7, 2 H–C(1′)); 5.14 (*t*, *J* = 7, H–C(2′)); 6.47 (*t*, *J* ≈ 2, H–C(6)). MS: 416 (16, $[M + 2]^+$), 414 (30, $M^{+\cdot}$), 203 (22), 189 (100), 151 (65), 57 (30), 43 (60). Anal. calc. for $C_{28}H_{46}O_2$ (414.67): C 81.10, H 11.18; found: C 81.43, H 11.36.

*[2-*methyl-^{3}*H,*^{14}*C]-2,3-Dimethyl-5-phytylcyclohexa-2,5-diene-1,4-dione* ([2-*methyl*-^{3}H,^{14}C]-**13**). The desired quinone [2-*methyl*-^{3}H,^{14}C]-**13** was prepared in analogy to the procedures for the nonlabelled compound, as described above using 48 mCi $^{14}CO_2$ and an excess of Li^{3}H$_4$. Starting with 3.5 mmol of **14**, 1.1 mmol of [2-*methyl*-^{3}H,^{14}C]-**13** were obtained; chem. purity 97%, radiochemical purity 98%, spec. act. ^{14}C: 16.5 mCi/mmol ^{3}H: 37 mCi/mmol, ^{3}H/^{14}C: 2.2.

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