A new vitamin E analogue more active than α-tocopherol in the rat curative myopathy bioassay

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Vitamin E owes its biological effects to its antioxidant activity. Kinetic and mechanistic studies on phenolic antioxidants in vitro have led us to design and synthesize *all-rac*-2,4,6,7-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-5-hydroxy-3,4-dihydrobenzofuran, **3**. In the rat curative myopathy bioassay the acetate of this compound has 1.5–1.9 times the bioactivity of *all-rac*-α-tocopherol acetate. This represents the first time that a rationally designed synthetic 'vitamin' has been found to have more activity in vivo than the corresponding natural vitamin.

Vitamin E potency *Benzofuran analog* *Pyruvate kinase* *Myopathy bioassay*

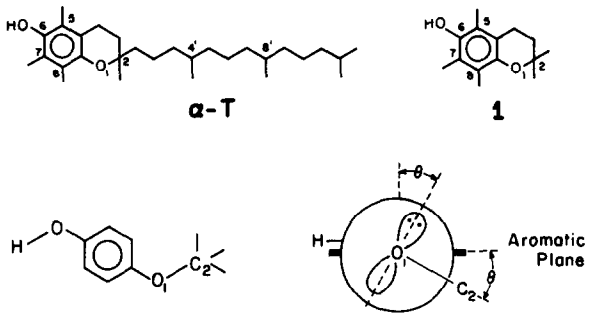
1. INTRODUCTION

Vitamin E, a mixture of four phenols (ArOH) called tocopherols, is believed to owe its biological effects to its antioxidant activity [1] which allows it to inhibit peroxidation in vivo by trapping chain carrying peroxyls (ROO·) [2,3].

$$ROO\cdot + ArOH \xrightarrow{k_1} ROOH + ArO\longrightarrow \quad (1)$$

α-Tocopherol (α-T) is biologically the most active of the four tocopherols [1]. It is also the most active at trapping peroxyls in vitro [4]. Indeed, we found that the rate constant for ROO· trapping, k_1, was higher for α-T ($k_1^{\alpha\text{-T}} = 3.2\times10^6$ $M^{-1}\cdot s^{-1}$) and related compounds, e.g. **1** ($k_1^1 = 3.8\times10^6$ $M^{-1}\cdot s^{-1}$), than for any other phenols then known [4]. This was explained in stereoelectronic terms with reference to the dihedral angle, θ ($\simeq 17°$ for **1**) [4] between the p-type lone pair orbital on O_1 and a perpendicular to the aromatic plane, an angle which is equal to the dihedral angle between the O_1–C_2 bond and the aromatic plane [4].

Since antioxidant activity (i.e. k_1) should increase as θ decreases we predicted that appropriate dihydrobenzofurans would be even better antioxidants than α-T and **1**. Such proved to be the case for **2** ($k_1^2 = 5.4\times10^6$ $M^{-1}\cdot s^{-1}$) [5] and the question was raised as to whether the α-T analogue, i.e. **3**, would have greater activity than α-T in vitro and in vivo [5]. We have now synthesized *all-rac*-**3**. We find that its in vitro ability to trap ROO· is greater than that of α-T by a factor of 1.47 ($k_1^3 = 4.7\times10^6$ $M^{-1}\cdot s^{-1}$, hence $k_1^3/k_1^{\alpha\text{-T}} = 4.7\times10^6/3.2\times10^6 = 1.47$). We therefore synthesized *all-rac*-**3**-acetate. We report herein the results of an in vivo bioassay of this compound comparative to that of



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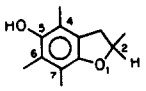
all-rac-α-T-acetate.



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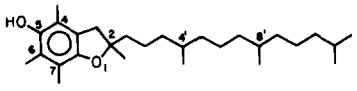

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2. MATERIALS AND METHODS

all-rac-**3** was synthesized by a Wittig reaction between 2-formyl-2,4,6,7-tetramethyl-5-benzyloxydihydrobenzofuran and 3,7,11-trimethyldodecyl bromide followed by hydrogenation. The identity of the compound (a colourless, oily liquid) was confirmed by elemental analysis (C and H), by ^{1}H and ^{13}C NMR, and by GC-MS of its trimethylsilyl ether. It was converted to the acetate which was then further purified by chromatography on silica gel. (The synthesis of this acetate by a different route was reported in 1948 but no supporting evidence (not even analysis for C and H) for its identity was provided [6]. Possibly the material did not have the ascribed structure since it was said to possess only a weak vitamin E activity [6].

The test chosen to measure relative vitamin E activities was the rat curative myopathy bioassay which was conducted essentially as described by Machlin et al. [7]. Male, weanling (21–22 days old), Sprague-Dawley rats from the NRCC specific pathogen-free facility were housed in individual stainless-steel wire mesh cages at $28 \pm 1°C$ (bioassays I and II) or $24 \pm 1°C$ (bioassay III), given tap water to drink and fed ad libitum a modified, vitamin E-free AIN 76A diet [8]. The higher levels of tocopherol-stripped fat used in the diets for bioassays II and III (see table 1) were accommodated by an appropriate reduction in diet sucrose. The diet employed in III was made selenium-deficient by substitution of the 3.5% AIN 76 salt mix with 4% of selenium-free 4164 salt mix [8]. When the rats' plasma pyruvate kinase (PPK) activities were $\geqslant$ 10-times the normal values of 0.2 ± 0.02 units/ml (12–14 weeks in bioassays I and II, 6 weeks in III), they were given one of three doses of the test compound daily for 4 days. In bioassay I test compounds were incorporated into an aqueous suspension of dimyristoyl lecithin liposomes (2 ml/kg body wt) and given by gastric intubation; in II and III they were dissolved in tocopherol-stripped corn oil and administered per os (200 μl/body wt) using a positive displacement pipette. Blood (0.5–1.0 ml) for PPK analysis was obtained 23–24 h after the last dose by cardiac puncture under halothane anaesthesia (5% in O_2, ~2 min). It was mixed with Na_2EDTA (1 mg/ml), chilled in ice and centrifuged at $12\,000 \times g$ for 1 min to sediment cells. The plasma was stored in ice and PPK was measured within 2 h essentially as described by Gutmann and Bernt [9]. The animals were killed after completion of these tests and lipid extracts from plasma and muscle tissue were analyzed. There was no statistically significant differences in the levels of α-T and **3** in either the plasma or the muscle of the animals in a particular bioassay group

In each bioassay the regression of PPK vs ln (dose) of the test compounds was computed by the method of least squares. Parallelism of the dose-response curves of each of the compounds in a given bioassay was confirmed by analysis of variance and the ratio of potencies of any two compounds was determined from the horizontal displacement between the corresponding least squares lines.

3. RESULTS AND DISCUSSION

The results are shown in fig.1 and table 1. Bioassay I, which was a preliminary assay with four animals per point, was also used to compare the bioactivity of (natural) 2*R*,4′*R*,8′*R*-α-T-acetate with that of (synthetic) *all-rac*-α-T-acetate. The biopotency of the natural isomer was found to be 1.29-times that of the *all-rac* material, which value lies within the range (1.16–1.62) reported in earlier replicate myopathy bioassays [7]. Very recently, it was reported that the rat myopathy test gives results for this pair of compounds (range 1.43–1.52) which are very similar to those obtained from the classical gestation-resorption assay conducted simultaneously on the same animals (range 1.28–1.41) [10]. The validity of the myopathy assay is further attested to by the fact that myopathy is observed in a variety of vitamin-E deficient animals, including man [10].

The differences in the slopes of the dose-response curves obtained in the different bioassays correspond to the differences in type and amount of fat in the various diets [7]. The virtual absence of selenium in the diet containing 10% corn oil

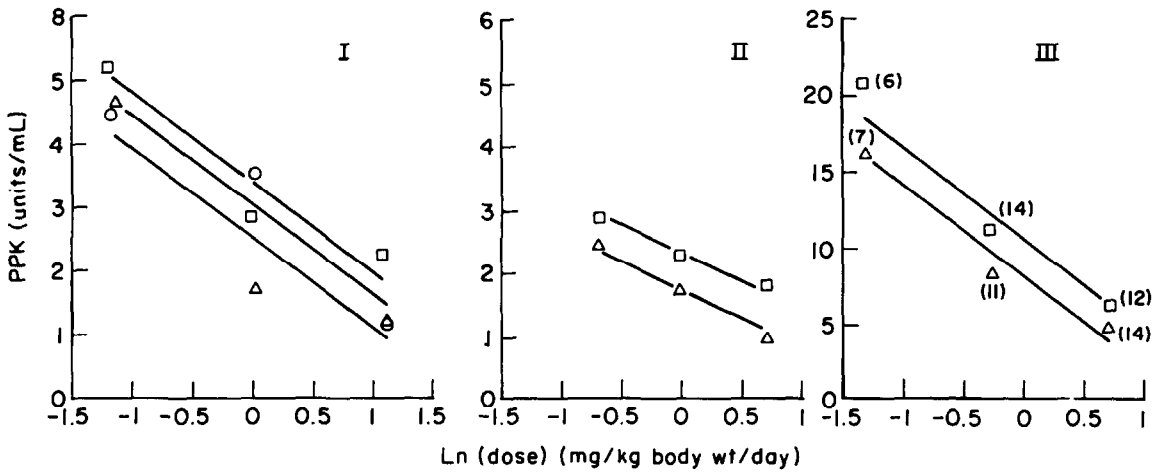

Fig.1. Plasma pyruvate kinase (PPK) levels of vitamin E-deficient rats vs administered dose of test compound: (□) *all-rac*-α-T-acetate, (△) *all-rac*-3-acetate, (○) *RRR*-α-T-acetate. Note that the vertical (PPK) scale in bioassay III differs from that in bioassays I and II. Bioassay I, 4 rats/point, initial PK level (±SE for 36 rats) 6.00±0.57 units/ml; II, 13 rats/point, no initial PK level but one week before test PK level for 6 rats was 3.04±0.47 units/ml; III, number of rats/point in parentheses, initial PK level (64 rats) 15.46±0.37 units/ml. Range of doses: I, 0.3, 1.0 and 3.0 mg/kg body wt per day; II and III, 0.5, 1.0 and 2.0 mg/kg body wt per day. Rats on a standard vitamin E-containing diet have PPK levels of ~0.2.

Table 1

Potency ratio of *all-rac*-3-acetate relative to *all-rac*-α-T-acetate

Bioassay	Source of dietary fat	Potency ratio	Confidence limits (95%)	Parallel slope[a]
I[b]	5% corn oil	1.86	0.78–4.45	−1.39
II	10% lard	1.93	1.10–3.38	−0.90
III[c]	10% corn oil	1.49	1.04–2.12	−6.10
	Average	1.76[d]		

[a] Slope of least-squares line fitted to each set of points. PPK units/ml/ln (mg/kg body wt/day)
[b] For 2*R*,4′*R*,8′*R*-α-T-acetate/*all-rac*-α-T-acetate: potency ratio = 1.29; 95% confidence limits = 0.55–3.01
[c] Selenium-deficient diet
[d] Error ± 0.18 is average of residuals

(bioassay III) produced a very marked acceleration in the rate of appearance of the myopathic condition and yielded the steepest dose-response curves ever reported. In each of the three bioassays the *all-rac*-3-acetate was found to be more effective in reducing PPK than *all-rac*-α-T acetate: mean potency ratio = 1.76, range 1.49–1.93. This in vivo potency ratio is rather similar to the ratio of 1.47 found for the in vitro antioxidant activities of the free phenols as determined from their k_1 values. This similarity (combined with the fact that in related sets of experiments the levels of α-T and 3 in plasma and muscle did not differ significantly) we take to provide strong additional support for the traditional view that vitamin E owes its biological effects to its in vivo antioxidant effects.

The measured biopotency of *all-rac*-3-acetate implies that this compound actually has slightly more vitamin E activity than (natural) *RRR*-α-T-acetate. To our knowledge, this represents the first time that in vitro chemical kinetic studies and the methods of physical organic chemistry have been used in vitamin research to design a compound which, in one test at least, 'improves' upon nature.

We have recently synthesized 2*R*,4′*R*,8′*R*-3-acetate and 2*S*,4′*R*,8′*R*-3-acetate and are currently testing their biopotencies.

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