Further studies of a new vitamin E analogue more active than α-tocopherol in the rat curative myopathy bioassay

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The bioactivities of the acetates of 2*R*,4′*R*,8′*R*- and 2*S*,4′*R*,8′*R*-2,4,6,7-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-5-hydroxy-3,4-dihydrobenzofuran (*RRR*- and *SRR*-**1**-Ac) have been measured in the rat curative myopathy bioassay and compared with the *RRR* and *SRR* stereoisomers of α-tocopheryl acetate (*RRR*- and *SRR*-**2**-Ac). Each stereoisomer of **1** is only slightly more active than the corresponding stereoisomer of **2** (*RRR*-**1**-Ac/*RRR*-**2**-Ac = 1.10, *SRR*-**1**-Ac/*SRR*-**2**-Ac = 1.16). This finding contrasts with our earlier finding [(1986) FEBS Lett 205, 117–120], confirmed in the present study, that *all-rac*-**1**-Ac is 1.5–1.9 as active as *all-rac*-**2**-Ac. We suggest that the stereochemistry (*S* vs *R*) at the 4′ and 8′ tail carbons is of less biological importance in **1** than in **2**

Vitamin E analogue, Rat curative myopathy assay, Peroxyl radical; Antioxidant

1. INTRODUCTION

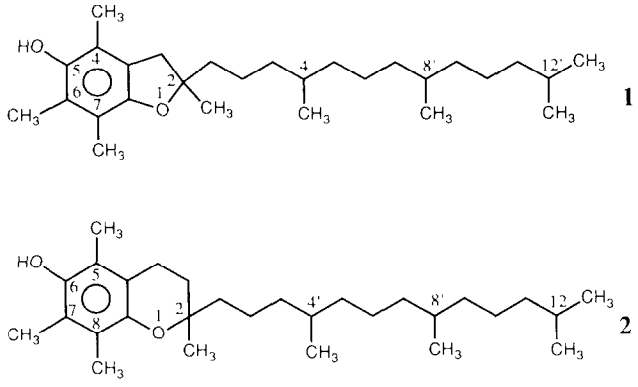
In 1986 we reported the synthesis of *all-rac*-2,4,6,7-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-5-hydroxy-3,4-dihydrobenzofuran, **1**, and showed that in the rat curative myopathy bioassay this compound had 1.5–1.9 times the vitamin E activity of *all-rac*-α-tocopherol, **2**, when both compounds were dosed as their acetates [1]. The greater biopotency of **1** was ascribed to the fact that it reacts in vitro with the peroxyl radicals (ROO·) which are responsible for lipid peroxidation with a rate that is 1.47 times the rate at which **2** reacts with ROO·, i.e., $k_1^1/k_1^2 = 1.47$,

$$\mathrm{ROO^{\cdot}} + \mathrm{ArOH} \xrightarrow{k_1} \mathrm{ROOH} + \mathrm{ArO^{\cdot}} \qquad (1)$$

this difference in reactivity having been rationalized on stereoelectronic grounds [1–3]. Of course, the situation in vivo *must* be more complex. Thus, it is well known that *all-rac*-**2** has less bioactivity than the 'natural' stereoisomer, 2*R*,4′*R*,8′*R*-**2** (*RRR*-**2**), the currently accepted ratio of bioactivities being 1.0:1.36 [4] (though in man the ratio is closer to 1:2 insofar as retention in plasma following a dose of a 1:1 mixture of the two forms is concerned (G. Burton and K. Ingold, unpublished results)). In fact, all 7 of the 'unnatural' stereoisomers of **2** have been shown to have less bioactivity than *RRR*-**2** [5] in the rat resorption-gestation bioassay despite the fact that their k_1 values in vitro must be identical. In this article we again employ the rat curative myopathy bioassay [6], which has been shown to correlate fairly well with the rat resorption-gestation bioassay for *all-rac*-**2** vs *RRR*-**2** [7], and for their acetates, i.e. *all-rac*-**2**-Ac vs *RRR*-**2**-Ac [8], and explore the influence of chirality at position 2 on the bioactivity of **1**, our reason being that it is known that it is the stereochemistry at the 2-position which is the major determinant of the bioactivity of **2** [5,6,9–14].

2. MATERIALS AND METHODS

all-rac-**1** was synthesized as described previously [1]. 2*R*,4′*R*,8′*R*-**1** (*RRR*-**1**) and 2*S*,4′*R*,8′*R*-**1** (*SRR*-**1**) were also synthesized as describ-



1

2

Scheme 1.

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ed previously [15]. These three compounds were converted to their acetates and further purified by chromatography on silica gel

The rat curative myopathy bioassay, which is based on the reduction of the highly elevated plasma pyruvate kinase (PK) activities of vitamin E deficient rats, was conducted essentially as described previously [1,6]. Male weanling (21–22 days old) Sprague-Dawley rats from the NRCC specific pathogen free facility were housed individually in stainless steel wire mesh cages at 21–23°C and with a 12:12 h light:dark cycle. Tap water and vitamin E-free diet were provided ad libitum. All three diets employed (see Table I) were modifications of the AIN 76 formulation [16] and contained 10% tocopherol-stripped corn oil (with 0.02% BHT) instead of 5% corn oil and had menadione concentrations of 500 μg/kg. Diet I contained 4% of the selenium-free 4164 salt mix (in place of the 3.5% AIN 76 salt mix) and sucrose was reduced from 50% to 44.5%. Diet II contained the 3.5% AIN 76 salt mix but had the sucrose reduced from 50% to 20% and the starch increased from 15% to 40%. Diet III was identical to diet II except that one-quarter of the starch was replaced with dyetrose (a selectively depolymerized corn starch) to permit pelleting. Diets I and II were purchased from ICN Biochemicals [16] and diet III from Dyets Inc., Bethlehem, PA. Vitamin A levels were formally the same in all three diets. The diets were fed for 16 weeks prior to use of the animals in the vitamin E bioassays.

Each bioassay normally employed 36 rats, each receiving one of three doses of either of two test compounds (i.e. 18 rats per compound and 6 per dose level) daily for 4 days. On day 1, before dosing, and on day 5 (23–24 h after the last dose) blood (0.5–1.0 ml) was obtained by cardiac puncture under brief halothane anesthesia (5% in O_2, 1–2 min), mixed with Na_2EDTA (1 mg/ml), chilled on ice and centrifuged at 8000 × g for 1 min to sediment cells. The plasma was retained and stored on ice until assayed (within 1–1.5 h) for PK activity, essentially as described by Gutman and Bernt [17]. The rats were ranked in order of initial (day 1) plasma PK and divided sequentially into 6 groups. The 6 rats in each group were then randomly assigned to receive one of the three doses of either test compound. The test compounds were dissolved in tocopherol-stripped corn oil and administered per os (250 μl/kg body weight) with a positive displacement pipette. After blood sampling on day 5 the animals were killed by exposure to gaseous CO_2.

For each bioassay the linear regression of plasma PK activity (units/ml) vs ln(dose) of test compound was computed by the method of least squares and, provided that parallelism of the dose-response lines was not statistically rejected by analysis of variance, a common slope was calculated and applied to the regressions. The ratio of potencies of the two test compounds was then computed from the horizontal displacement of the two lines.

3. RESULTS AND DISCUSSION

The results of 7 bioassay experiments (2 of which employed twice the normal number of rats), in terms of potency ratios for each of the pairs of compounds tested are given in Table I and some typical plots of plasma PK activity vs ln(dose) are shown in Fig. 1.

Our present results confirm our earlier finding [1] that *all-rac*-**1**-Ac has significantly greater activity in the rat curative myopathy bioassay than synthetic vitamin E, i.e. than *all-rac*-**2**-Ac (see test 4). Moreover, the present potency ratio for these two stereoisomeric mixtures, viz. 1.55, is in excellent agreement with the previously reported ratios from three separate assays, viz. [1], 1.49, 1.86 and 1.93, average 1.76.

In contrast to the results obtained with *all-rac*-**1** and -**2** acetates stand the results obtained with the pure stereoisomers. Thus, the directly measured potency ratio for the *RRR*-**1**-Ac/*RRR*-**2**-Ac pair is 1.10 (test 1) while the potency ratios that may be calculated for this pair by combining the data from tests 2 and 5 and from tests 6 and 7 are 1.49 and 1.09, respectively. Similarly, the directly measured potency ratio for the *SRR*-**1**-Ac/*SRR*-**2**-Ac pair is 1.16 (test 3) while the potency ratios which may be calculated for this pair by combining the data from tests 2 and 7 and from tests 5 and 6 are 1.55 and 1.13, respectively. Although both the directly measured and indirectly calculated potency ratios for the *RRR* pair and the *SRR* pair are not, statistically speaking, significantly different from 1.0 (see Table I) the total weight of evidence is that *RRR*-**1**-Ac and *SRR*-**1**-Ac are ~1.1–1.5 times as active as the corresponding stereoisomers of α-tocopherol acetate. We therefore conclude that the statistically significantly greater activity of all *all-rac*-**1**-Ac relative to *all-rac*-**2**-Ac (Table I and [1]) is most likely to be due to a significantly higher activity of some (or all) of the

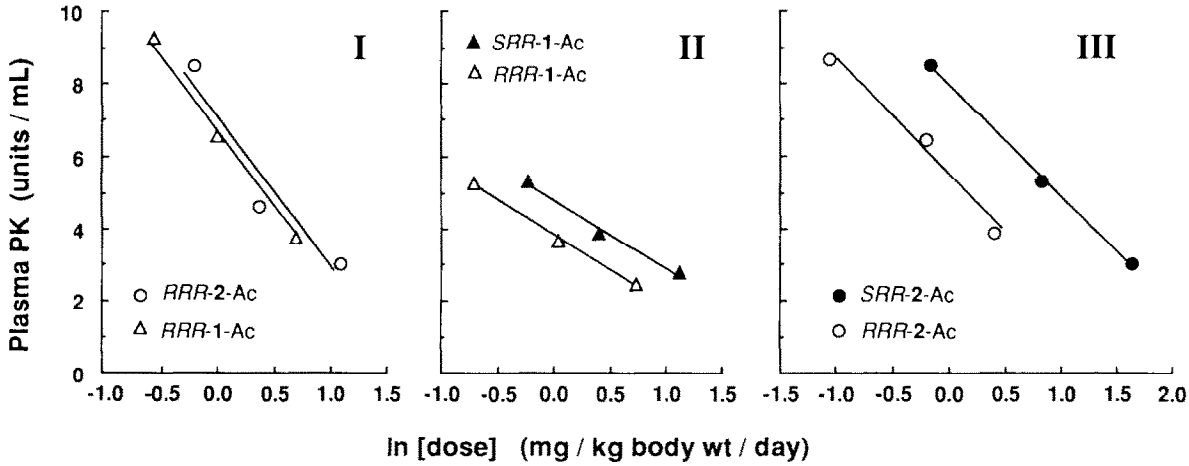

Fig. 1. Dose-dependence of the reduction in plasma pyruvate kinase (PK) levels measured on day 5 in vitamin E deficient rats after administration once daily for 4 consecutive days of I. *RRR*-**2**-Ac (○) or *RRR*-**1**-Ac (△) (test 1, assay 1); II: *RRR*-**1**-Ac (△) or *SRR*-**1**-Ac (▲) (test 5, assay 1); and III: *RRR*-**2**-Ac (○) or *SRR*-**2**-Ac (●) (test 7, assay 1). The data points displayed in each graph are the means of data from 6 animals.

Table I

Potency ratio of various pure stereoisomers and mixtures of stereoisomers of the acetates of **1** and **2** in reducing plasma pyruvate kinase in the rat curative myopathy bioassay

Test	Pair of compounds		Potency ratio, A:B[a]				Diet
	A	B	Assay 1	Assay 2	Assay 3	Average	
1	*RRR*-**1**-Ac	*RRR*-**2**-Ac	1.09^{ns}	0.96^{ns}	1.24^{ns}	1.10	I
2	*SRR*-**1**-Ac	*RRR*-**2**-Ac	0.76^{ns}			0.76	I
3	*SRR*-**1**-Ac	*SRR*-**2**-Ac	$1.16^{ns,d}$			1.16	I
4	*all-rac*-**1**-Ac	*all-rac*-**2**-Ac	$1.55^{c,d}$			1.55	III
5	*SRR*-**1**-Ac	*RRR*-**1**-Ac	0.60^{c}	0.43^{c}		0.51	II
6	*SRR*-**2**-Ac	*RRR*-**1**-Ac	0.42^{c}	0.47^{c}		0.45	II
7	*SRR*-**2**-Ac	*RRR*-**2**-Ac	0.44^{b}	0.56^{c}	0.46^{c}	0.49	I

[a] The superscripts ns, b and c indicate the degree of significance, $P > 0.05$, $P < 0.05$ and $P < 0.01$, respectively, that the test pair of compounds do not differ in potency

[d] Double assay with 36 rats per test compound rather than the usual 18 rats

stereoisomers of **1**-Ac relative to the corresponding stereoisomers of **2**-Ac. We prefer not to invoke some hypothetical synergism between the stereoisomers of '*all-rac*-**1**-Ac' although we note that 'synergism' has previously been invoked to explain relative bioassay results for **2**-Ac stereoisomeric mixtures and single stereoisomers which were analogous to those given in Table I [5,13,18]. It may, however, be significant that chirality differences in ^{13}C NMR spectra are less pronounced in stereoisomers of **1** than in stereoisomers of **2** [15] which suggests that the stereochemistry (*S* vs *R*) at the 4′ and/or 8′ carbons of **1** is of less biological importance than would appear to be the case for **2** [5].

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