# Antioxidant Activity of 2,6-Dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridines in Metal-Ion Catalyzed Lipid Peroxidation

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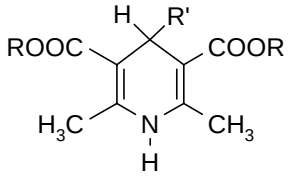
**Abstract**

TIRZITIS G., TIRZITE D., HYVONEN Z. (2001): **Antioxidant activity of 2,6-dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridines in metal-ion catalyzed lipid peroxidation**. Czech J. Food Sci., **19**: 81–84.

Antioxidants with 1,4-dihydropyridine structure were investigated as a less harmful alternative to synthetic phenolic antioxidants in liposomes under conditions simulating food storage. The antioxidant activities (AOA) of 2,6-dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridines possessing various side chain length alkyls ($CH_3$ - $C_{16}H_{33}$) in ester moiety were tested in transition metal-ion catalyzed liposome peroxidation and compared with AOA of Trolox™ and Probucol™. The compounds with $C_2H_5$ - $C_4H_9$ residues in the 3,5-position ester moieties exert the most pronounced AOA. The AOA of tested compounds is associated with their ability to incorporate into liposomes.

**Keywords**: 1,4-dihydropyridines; antioxidants; metal-ion catalyzed peroxidation

The 1,4-dihydropyridine (I, 1,4-DHP) derivatives, especially compounds unsubstituted in position 4 (I, R'=H), show AOA in homogenous lipid systems (TIRZITIS *et al*. 1988; ABDALLA *et al.* 1999).




I

The 2,6-dimethyl-3,5-diethoxycarbonyl-1,4-DHP (trade name Diludin) possesses considerable AOA in the stabilization of edible oils (KOUŘIMSKÁ *et al.* 1993) and exhibits synergistic properties with antioxidants, such as α-tocopherol (TIRZITIS *et al.* 1983) and 2,6-bis(tert-butyl)-4-hydroxytoluene (BHT) (TIRZITIS & KIRULE 1999). Unfortunately the insufficient solubility of Diludin in lipids limits its practical application. This limitation demanded the synthesis and investigation of more lipophilic Diludin homologues.

Most foods differ from refined oils and fats in that they are oil-in-water emulsions, where the aqueous phase contains hydrated proteins and carbohydrates (including dietary fibre), and on the other hand, also trace metals that catalyze oxidation. The 1,4-DHP has been less investigated in transition valence metal-ion catalyzed peroxidation. The aim of our study was to investigate the relationship between AOA and alkyl substituent (R) length in of increasing lipophilicity ranging from $C_1$ (methyl) to $C_{16}$ (hexadecyl) in the ester moiety of 1,4-DHP. This would help to choose the best compound for fat, oil and another lipid containing product stabilization studies.

We used metal-ion (ferrous and copper) catalyzed phospholipid liposome peroxidation. Liposomes belong to very important lipid systems, present in most foods. The AOA of tested I was compared with AOA of Probucol™ and Trolox™, which belong to relatively polar antioxidants. The study of incorporation of 1,4-DHP into phosphatidylcholine liposomes was carried out as well. The results could be useful for the stabilization of non-homogenous lipid products (margarines, salad dressings, creams, etc.).

## MATERIALS AND METHODS

Phospholipid (bovine brain extract) – Sigma Chemical Co., St.Louis, Mo, USA; phosphatidylcholine – Olaine Chemical-Pharmaceutical Plant, Olaine, Latvia; anthra-

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cene – Fluka AG, Germany. All other reagents and solvents – Aldrich, USA.1,4-DHP derivatives were synthesized in the Latvian Institute of Organic Synthesis according to Hantzsch procedure (SAUSINS & DUBURS 1988). Purity of compounds was > 98% (HPLC data).

***Antioxidant Activity Evaluation***: The iron ion ($Fe^{2+}$) catalyzed phospholipid liposome peroxidation was performed according to chain breaking reaction assay (GUTTERIDGE & QUINLAN 1992). Thus, 0.2 ml of liposomes (prepared from 5 mg of brain extract phospholipids in 1 ml of 0.1 M phosphate buffer, pH = 7.4), 0.2 ml of 0.1 M phosphate buffer (pH = 7.4), 0.01 ml of sample solution (Ia-d in ethanol; Ie-f in n-butanol; Ig in deionized water), 0.02 ml of 1 mM ferric chloride and 0.02 ml of 7.5 mM sodium ascorbate were incubated in 10 ml plastic test tubes (L.I.P. Ltd, UK) for 20 min at 37°C. After adding 0.5 ml of 25% (v/v) HCl, 0.5 ml of 1% TBA (2-thiobarbituric acid) and heating at 100°C for 15 min, subsequent cooling, adding 1.5 ml of 1-butanol, the reaction system was vortexed, spun down and the butanol extract absorbancy (A) read at 532 nm.

The copper ion ($Cu^{2+}$) catalyzed peroxidation was carried out according to (GUTTERIDGE 1983). Similarly as in the above-mentioned assay, 0.2 ml of liposomes, 0.2 ml of 0.025 M phosphate buffer (pH = 7.4), 0.01 ml of sample solution and 0.05 ml of 1 mmol cupric chloride were incubated at 37 °C for 1 h. The TBA reaction was carried out as in the previous assay. The TBA test data are used for AOA calculation:

$$AOA\ (in\ \%)=100 - \{[(A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100\}$$

Mean values of at least 3 repeated determinations are presented in Table 1. The standard deviation determined for Ib ($n$ = 6) was 10% of mean value. Due to the insufficient solubility of some 1,4-DHP derivatives it was not possible to express the AOA in values of $IC_{50}$.

***The Determination of 1,4-DHP Incorporation into Liposomes***: The fluorescence method (LAKOWITZ 1983) was used. Phosphatidylcholine liposomes were prepared in 0.01 M TRIS-HCl buffer (pH 7.4); concentration of lipid was 0.05 mg/ml. To 1 ml of vigorously stirred liposomes 5 µl of 10 mmol anthracene in ethanol, and then 5 µl of 10 mM 1,4-DHP solution in ethanol, were added. Anthracene fluorescence was measured using the fluorometer produced by "Hitachi-850" (Tokyo, Japan) in a 1 mm cell oriented at $45^0$ to the excitation beam (excitation at 340 nm and emission at 382 nm). The incorporation was expressed as the ratio of ln ($F_0$ /F), where $F_0$ and F are intensities of anthracene fluorescence in the absence and in the presence of 1,4-DHP, respectively. Mean values of triplicate analyses are presented in the Table 1. The standard deviation determined for Ib ($n$ = 5) was 5% of mean value.

## RESULTS AND DISCUSSION

The AOA comparison of tested compounds is shown in Table 1. The compounds Ib (Diludin) and Id are most active. The compound Id possesses approximately the same AOA as the compound Ib in an iron ion ($Fe^{2+}$) catalyzed test-system. In this assay both compounds (Ib and Id) possess higher AOA than Trolox™ and Probucol™. In a copper catalyzed test-system the compound Id is less active than the compound Ib. To reach the approximately equal AOA a fivefold concentration of the compound Id must be used. The AOA reduction of the

Table 1. Antioxidant activity of 2,6-dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridine (I) AOA in metal-catalyzed peroxidation of phospholipid liposomes and incorporation into phosphatidylcholine liposomes

| I | | $Fe^{2+}$ catalyzed assay | | $Cu^{2+}$ catalyzed assay | | Incorporation into liposomes |
|---|---|---|---|---|---|---|
| Compound code | R | concentration* [mM] | AOA [%] | concentration* [mM] | AOA [%] | $lnF_0/F$ |
| Ia | $CH_3$ | 5 | 83 | 5 | 90 | 0.12 |
| Ib | $C_2H_5$ | 0.5 | 88 | 1 | 97 | 0.29 |
| Ic | Ib; $R^{'}=CH_3$ | 5 | 62 | 5 | 40 | 0.19 |
| Id | $C_4H_9$-n | 1 | 100 | 5 | 100 | 0.43 |
| Ie | $C_{10}H_{19}$ | 5 | 8 | 5 | 0 | 0.06 |
| If | $C_{16}H_{33}$ | 5 | 3 | 5 | 0 | 0.03 |
| Ig | $CH_2COONa$ | 5 | 3 | 5 | 15 | 0.03 |
| Probucol | | 2.5 | 76 | 2.5 | 90 | n.d.** |
| Trolox | | 5 | 54 | 2.5 | 82 | n.d.*** |

*concentration of tested compound in the added solution

**not determined (impossible due to absence of Probucol and Trolox UV-spectra overlapping with anthracene UV-spectra)

compound Ic (methyl substituent in position 4), as compared with the parent compound Ib, is probably caused by sterical hindrance of the methyl group on the parent 1,4-DHP ring. The results show that 1,4-DHP bearing the alkyl radicals R = $C_2H_5$ or $C_4H_9$ (Ib, Id, respectively) in substituents of position 3, 5 exhibits the optimum of AOA in both test-systems used. Similarly the optimum of 1,4-DHP incorporation into liposomes was found for the compounds Ib and Id. The increased incorporation in the case of compound Id compared with compound Ib is in good agreement with the increase of lipophilicity of the compounds mentioned. The lipophilicity of compounds Ib and Id, determined as partition coefficients lgP in n-octanol-water, is 3.6 and 5.1, respectively (SHATZ *et al.* 1985).

The decrease of AOA and incorporation into liposomes for I with long alkyl chains (R > $C_4H_9$) probably is due to its tendency to self-aggregation (TIRZITE *et al.* 1999). This self-aggregation is especially observed for If (R = $C_{16}H_{33}$). We suggest that this effect decreases the amount of 1,4-DHP available for its incorporation into liposomes and consequently the concentration of the antioxidant in liposomes.

The data obtained would be important for disperse lipid system stabilization as it is well known that applications of liposomal encapsulation technique of food ingredients in food industry increases (GIBBS & KERMASHA 1999).

The obtained results reveal that the AOA of antioxidants in disperse systems, at least in 1,4-DHP derivatives I, depend on electron and/or hydrogen donating properties of compounds and the ability of incorporation into autoxidizing/peroxidizing particles. Molecules that cannot incorporate into liposomes can influence the autoxidation/peroxidation process only to a certain extent. This presumption is confirmed by the insignificant AOA of the compound Ig (R = $CH_2COONa$). Although this compound possesses pronounced radical scavenging activity (RUBENE *et al.* 1982), its incorporation into negatively charged liposomes is diminished due to electrostatic repulsion of negatively charged $COO^-$ groups of the liposome surface.

Regarding the practical use of 1,4-DHP derivatives tested in this study we suggest that it could be useful to continue research in the use of the compound Id for the stabilization of oil-in-water systems. It is necessary to underline that 1,4-DHP is mainly preferred as a prospective lipid antioxidant due to its very low toxicity. Thus, acute toxicity ($LD_{50}$) of Diludin (compound Ib) is about 10 000 mg/kg (mice, per os) and long term administration of this compound to experimental animals does not cause any toxic effect (GILLER *et al.* 1970).

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**Souhrn**

TIRZITIS G., TIRZITE D., HYVONEN Z. (2001): **Antioxidační aktivita 2,6-dimethyl-3,5-dialkoxykarbonyl-1,4-dihydropyridinů při oxidaci lipidů katalyzované ionty těžkých kovů**. Czech J. Food Sci., **19**: 81–84.

Antioxidanty s 1,4-dihydropyridinovou strukturou, představující méně závadnou alternativu fenolických antioxidantů, byly zkoumány za podmínek obdobných skladovaným potravinám. Antioxidační aktivity 2,6-dimethyl-3,5-dialkoxykarbonyl-1,4-dihydropyridinů s různými alkylovými substituenty o 1–16 atomech uhlíku v esterových skupinách byly stanoveny v liposomech za přítomnosti kovů o přechodném mocenství a byly srovnány s účinností antioxidantů Trolox a Probucol. Nejvýznačnější antioxidační aktivitou se vyznačovaly deriváty s alkyly o 2–4 atomech uhlíku v poloze 3 a 5. Antioxidační aktivita zkoumaných látek byla závislá na jejich schopnosti vniku do struktury liposomů.

**Klíčová slova:** 1,4-dihydropyridiny; antioxidanty; oxidace katalyzovaná těžkými kovy; jedlé oleje

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