# SYNTHESIS, ANTIOXIDANT ACTIVITY AND MEMBRANE BINDING OF 4,5,6-SUBSTITUTED 2-METHYLTHIO-3-CYANO-1,4-DIHYDROPYRIDINES

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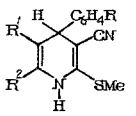
Continuing our research on the synthesis and properties of the 2-alkylthio-1,4-dihydropyridines [1, 2], we have obtained new 4,5,6-substituted 2-methylthio-3-cyano-1,4-dihydropyridines, and determined their antioxidant activity (AOA) and membrane binding level. AOA has been extensively studied in the 2,6-dimethyl-3,5-dialkoxycarbonyl-1,4-dihydropyridine series [3, 4]. Antioxidant and hepatoprotective properties have also been found in related compounds such as the 1,4-dihydropyridine-2(3H)-thiones [5, 6, 7]. Investigation into the AOA and membranotropic properties of the newly-synthesized 2-alkylthio-1,4-dihydropyridines is vital in the continuing search for physiologically active substances among the derivatives of 1,4-dihydropyridines (DHP), since the peroxide oxidation of lipids and the regulation of this process by membranotropic compounds is of considerable importance in a number of diseases.

4,5,6-Substituted 2-methylthio-3-cyano-1,4-DHP (Ia, Ib, Id-g) were obtained in high yields by alkylating readily available piperidine 3-cyano-1,4-dihydropyridine-2-thiolate with methyl iodide or dimethyl sulfate. Compound Ic was synthesized either by alkylating 1,4-dihydropyridine-2(3H)-thione, or dehydrating the appropriate 6-hydroxy-1,4,5,6-tetrahydropyridine.



Ia-h

R=H(Ia,d, g), n-Cl(Ib, c, e, f, g); $R^1$=H(Ia, b), COOEt(Ic, d, e), COOMe(If), Ac(Ig, h); $R^2$=Ph(Ia, b, c), *Me*(Id, e, f, g, h)

The structure of compounds I was verified by spectroscopy. The cyano group absorption band at 2190-2202 $cm^{-1}$ and the $\nu_{CO}$ band of the alkoxycarbonyl substituent at 1685-1702 $cm^{-1}$ were the most characteristic in the IR spectra of these compounds.

In the PMR spectra the 4-H and 5-H proton signals in the form of a doublet with $^3J_{H_4H_5}$ = 5.0 Hz were the most distinctive for compounds Ia and Ib, while the 4-H proton signals appearing as a singlet were most characteristic in the case of compounds Ic-h.

The UV spectra of DHP Ia and Ib exhibited a longwave absorption at 339-342 nm, which in the case of the 5-substituted compounds underwent a bathochromic shift to 352-357 nm. This is explained by increased conjugation in the molecule.

AOA was determined using two methods: The first involved recording polarographically the rate at which oxygen was consumed by lecithin liposomes prepared in the manner reported in a previous work [8] in the presence of a peroxide oxidation catalyst, namely, methemoglobin; the second comprised the auto-oxidation of a mixture of $\beta$-carotene and lineolate in a water-dispersion system [5, 6]. As the data in Table 1 show, there was a correlation (r = 0.97) between the AOA values obtained using these two methods.

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TABLE 1. Antioxidant Activity of Dihydropyridines Ia-h

| Compound | AOA ($V_0/V_i$) $c_i=5\cdot10^{-6}$ M | AOA ($\tau/\tau_0$) $c=3.7\cdot10^{-6}$ M |
|---|---|---|
| Ia | 5,3 | 3,0 |
| Ib | 2,5 | 2,3 |
| Ic | 0,85 | 1,0 |
| Id | 0,7 | 1,0 |
| Ie | 1,2 | 1,0 |
| If | 0,6 | 1,0 |
| Ig | 1,2 | 1,3 |
| Ih | 3,3 | 2,8 |
| 2,6-dimethyl-3,5-diethoxy-carbonyl-1,4-dihydropyridine (diethone) | 2,3 | 2,7 |
| Ionol | 11.5 | 11.3 |

TABLE 2. Absorbance and Fluorescence Spectral Data for 2-Methylthio-3-cyano-1,4-dihydropyridines in Ethanol and in Liposomes

| Compound | $\lambda$ absorp. eth. | $\lambda$ fluor. eth. | $\lambda$ fluor. lip. |
|---|---|---|---|
| I a | 339 | 408 | 414* |
| I b | 342 | 409 | 410** |
| I d | 355 | 430 | 437 |
| Id Analogue (2-$CH_3$) | 344 | 412 | 423 |
| I e | 356 | 426 | 435** |
| I g | 368 | 444 | 440 |
| I h | 368 | 446 | 445** |
| Diludin (diethone) | 371 | 453 | 455 |
| Foridon (riodipin) | 363 | 428 | 430 |

*Narrow band.

**Broad band.

Membrane binding, i.e., the ability to incorporate into a lipid bilayer, was determined by fluorescence spectroscopy, measuring the extent to which the test compounds quenched the characteristic fluorescence of an anthracene probe [14]; phosphatidylcholine liposomes were used as the membrane model [8].

As was to be expected, the findings (Table 1) show that AOA was more pronounced in the 2-methylthio-3-cyano-1,4-DHP that were unsubstituted at position 5 (compounds Ia and Ib). On the whole introducing electron-acceptor alkoxycarbonyl groups into position 5 of the 1,4-DHP ring reduced AOA. This was also in line with the reactivity of these derivatives in oxidation reactions (derivatives substituted at position 5 are more stable to oxidation). The presence of an electron acceptor (Cl) in the 4-phenyl group (compound Ib) also decreased AOA as compared with that of Ia. This lent support to the fact that AOA is dependent on the nature of the substituents, which was established in a previous work involving 2,6-dimethyl-2,5-dicarbonyl-1,4-DHP [3, 4].

In marked contrast to the above, however, compound Ih with its 5-acetyl group had a slightly higher AOA than the Ib analogue that was unsubstituted at position 5. The 5-acetyl derivative Ig also displayed antioxidant activity with stabilization of $\beta$-carotene. More detailed investigations into the structural peculiarities and chemical properties of these compounds were required to explain this phenomenon, and these are currently being undertaken.

As is clear from the characteristic fluorescence spectra (Table 2), the parameters for the DHP test compound group only differed by a few nanometers for ethanol and the liposomes, indicating that the polarity of the medium had little effect. Shifts were observed toward both the long and shorter wave directions, spectral bands were comparatively broad, and luminescence was seen with a quantum efficiency lower than that of riodipin (data not cited). All of this made it difficult to discern an overall trend in the electronic effect that the substituents have on the shape and position of fluorescence spectral maxima.

Since the fluorescent anthracene probe, by the nonpolar nature of the compounds, still gravitated to the surfaces of the lipid bilayer [14], and, as Table 3 shows, anthracene fluorescence was quenched by the test compounds, the latter are probably localized on the bilayer surface. As I, for the compounds varied within quite a broad range (0.21 → 0.54), there was a

TABLE 3. Binding of 2-Methylthio-3-cyano-1,4-dihydropyridines with Liposomes (from anthracene fluorescence quenching data)

| Compound | I=Ln ($F_0$/F) |
|---|---|
| Ia | 0,33 |
| Ib | 0,21 |
| Id | 0,48 |
| Id Analogue (2-$CH_3$) | 0,41 |
| Ie | 0,54 |
| Ig | 0,33 |
| Ih | 0,56 |
| Diludin (diethone) | 0,48 |
| Foridon (riodipin) | 0,62 |

TABLE 4. Physical Properties of the 2-Methylthio-3-cyano-1,4-dihydropyridines (I)

| Compound | Yield, % | mp, °C | Empirical formula |
|---|---|---|---|
| Ia | 66 | 147—149 | $C_{19}H_{16}N_2S$ |
| Ib | 60 | 138—140 | $C_{19}H_{15}N_2ClS$ |
| Ic | 74* | 135—137 | $C_{22}H_{19}N_2ClO_2S$ |
| | 54** | 134—136 | |
| Id | 90 | 123—125 | $C_{17}H_{18}N_2O_2S$ |
| Ie | 82 | 116—118 | $C_{17}H_{17}N_2ClO_2S$ |
| If | 62 | 172—173 | $C_{16}H_{15}N_2ClO_2S$ |
| Ig | 85*** | 182—184 | $C_{16}H_{16}N_2OS$ |
| Ih | 80*** | 168—169 | $C_{16}H_{15}N_2ClOS$ |

*Method A.

**Method B.

***Synthesized by method [1].

corresponding change in their lipophilic affinity, i.e., the depth of submersion in the bilayer. There was no parallel to be seen here with AOA. In examining the effect of the substituent, it was found that an n-Cl-phenyl substituent in position 4 increased the lipophilic affinity as compared to the phenyl substituent compound (see Ie and Id), but with the proviso that there was a substituent at position 5. When an H atom was present at position 5, however, the affinity actually decreased (see Ib and Ia). Thus, position 5 is of decisive importance both for AOA and lipophilic affinity. The introduction of a $SCH_3$ substituent into position 2 instead of the $CH_3$ produced a slight increase in lipophilic affinity. When the $COOC_2H_5$ moiety at position 5 was replaced by a $COCH_3$ (see Id and Ig), the affinity decreased. Although all the test substances could be classed as membranotropic, lipophilic compounds, none of them rivaled riodipin (foridon) in terms of lipophilic affinity.

In summary, although the AOA of the 2-methylthio-3-cyano-1,4-DHP derivatives was lower than that of the widely-used antioxidant ionol, it stood in comparison with and, in some cases, even surpassed that of 1,4-DHP series antioxidant diethone.

In view of the relative ease of accessibility of the 2-methylthio-3-cyano-1,4-DHP, coupled with their stability and low toxicity, further research into these compounds may be worthwhile.

## EXPERIMENTAL (CHEMICAL)

IR spectra were taken in Vaseline on a Perkin–Elmer 580 B (UK), UV spectra in ethanol on a Specord UV-vis (Germany), and PMR spectra in DMSO-$d_6$ on a WH 90/DC (Germany) at a frequency of 90 MHz, internal standard TMS. The principal physicochemical properties of the synthesized substances are cited in Table 4. Elemental analysis findings were in agreement with calculated values.

Compounds Ig and Ih were synthesized using the method outlined in an earlier report [1].

**Methylthio-4-phenyl(p-chlorophenyl)-6-phenyl-3-cyano-1,4-dihydropyridines (Ia, Ib).** A mixture of 3.75 g (10 mmoles) of piperidine 4,6-diphenyl-3-cyano-1,4-dihydropyridine-2-thiolate [11] and 1.4 ml (15 mmoles) of dimethyl sulfate in 20 ml of abs. ethanol were stirred for 30 min at room temperature and cooled to 0°C. After 2 h the resultant precipitate was filtered off and washed with cold ethanol and water. Yield 2.0 g (66%) of compound Ia with mp 147-149°C (from ethanol). UV spectrum, $\lambda_{max}$, nm: 244, 271, 339. IR spectrum, $\nu$, $cm^{-1}$: 2188 (C≡N); 3290 (N−H). PMR spectrum, $\delta$, ppm: 8.93 s (N−H); 7.5-7.2 m ($2C_6H_5$); 5.06 d (5-H); 4.33 d (4-H); 2.50 s (S-$CH_3$).

Compound Ib was obtained in a similar way. UV spectrum, $\lambda_{max}$, nm: 244, 268, 342. IR spectrum, $\nu$, $cm^{-1}$: 2192 (C≡N); 3264 (N−H). PMR spectrum, $\delta$, ppm: 9.01 s (N−H); 7.5-7.2 m ($C_6H_5$ and n-$ClC_6H_4$); 5.06 d (5-H); 4.39 d (4-H); 2.52 s (S−$CH_3$).

**2-Methylthio-6-phenyl-4-(p-chlorophenyl)-5-ethoxycarbonyl-3-cyano-1,4-dihydropyridine (Ic).** A. A mixture of 3.97 g (10 mmoles) of 6-phenyl-4-(p-chlorophenyl)-5-ethoxycarbonyl-3-cyano-1,4-dihydropyridine-2(3H)-thione [12], 1.2 ml of piperidine and 1.5 ml (24 mmoles) of methyl iodide in 20 ml of abs. ethanol was heated for a short period on a water bath and cooled to 0°C. Two hours later the resultant precipitate was filtered off and washed with cold ethanol and water. Yield 3.04 g (74%) of compound Ic. UV spectrum, $\lambda_{max}$, nm: 254, 288 p, 357. IR spectrum, $\nu$, $cm^{-1}$: 1685 (C=O); 2197 (C≡N); 3245 (N−H). PMR spectrum, $\delta$, ppm (in $CDCl_3$): 6.19 s (N−H); 7.5-7.2 d and d ($C_6H_5$ and n-$ClC_6H_4$); 4.76 s (4-H), 3.83 and 0.82 q and t ($C_2H_5$); 2.49 s (S−$CH_3$).

B. A mixture of 1.29 g (3 mmoles) of 6-hydroxy-2-methylthio-6-phenyl-4-(p-chlorophenyl)-5-ethoxycarbonyl-3-cyano-1,4,5,6-tetrahydropyridine and 5 ml of conc. HCl in 5 ml of ethanol was heated for 30 min on a water bath and cooled. Then 10 ml of water was added and the precipitate was filtered off and recrystallized from ethanol. Yield 0.66 g (54%) of compound Ic.

**2-Methylthio-6-methyl-4-phenyl(p-chlorophenyl)-5-ethoxycarbonyl-3-cyano-1,4-dihydropyridines (Id, Ie).** A mixture of 3.86 g (10 mmoles) of piperidine 6-methyl-4-phenyl-5-ethoxycarbonyl-3-cyano-1,4-dihydropyridine-2-thiolate [13] and 2.0 ml (32 mmoles) of methyl iodide in 12 ml of abs. ethanol was heated for a short time on a water bath and cooled to 0°C. One hour later the resultant precipitate was filtered off and washed with cold ethanol and water. Yield 2.83 g (90%) of compound Id with mp 123-125°C (from ethanol). UV spectrum, $\lambda_{max}$, nm: 226, 281, 355. IR spectrum, $\nu$, $cm^{-1}$: 1702 (C=O), 2198 (C≡N), 3278 (N−H). PMR spectrum, $\delta$, ppm: 9.38 s (N−H); 7.4-7.0 m ($2C_6H_5$); 4.43 s (4-H); 3.90 and 1.00 qt and t ($C_2H_5$); 2.44 s (S−$CH_3$); 2.26 s (6-$CH_3$).

Compound Ie was obtained in a similar manner. UV spectrum, $\lambda_{max}$, nm: 222, 284, 356. IR spectrum, $\nu$, $cm^{-1}$: 1688 (C=O); 2202 (C≡N); 3380 (N−H). PMR spectrum, $\delta$, ppm: 9.40 s (N−H); 7.4-7.1 d and d (n-$ClC_6H_4$); 4.47 s (4-H); 3.89 and 1.02 q and t ($C_2H_5$); 2.44 s (S−$CH_3$), 2.27 (6-$CH_3$).

**2-Methylthio-6-methyl-4-(p-chlorophenyl)-5-methoxycarbonyl-3-cyano-1,4-dihydropyridine (If).** A mixture of 1.6 ml (20 mmoles) of methyl acetoacetate and 2.81 g (20 mmoles) of n-chlorobenzaldehyde in 10 ml of abs. ethanol and 0.5 ml of piperidine was stirred for 5 min at room temperature. To this was added a mixture of 2.0 g (20 mmoles) of cyanothioacetamide in 10 ml of abs. ethanol and 1.5 ml of piperidine. After the mixture had been stirred for a further 30 min and 4.0 ml (64 mmoles) of methyl iodide had been added, it was heated for a short time on a water bath and cooled to 0°C. Three hours later the resultant precipitate was filtered off and washed with cold ethanol and water. Yield 4.15 g (62%) of compound If with mp 172-173°C (from ethanol). UV spectrum, $\lambda_{max}$, nm: 218, 240p, 284, 352. IR spectrum, $\nu$, $cm^{-1}$: 1700 (C=O); 2190 (C≡N); 3285 (N−H). PMR spectrum, $\delta$, ppm: 9.52 s (N−H); 7.4-7.1 d and d (n-$ClC_6H_4$); 4.54 s (4-H); 2.49 s (S−$CH_3$); 2.33 s (6-$CH_3$).

## EXPERIMENTAL (BIOCHEMICAL)

Antioxidant activity of the compounds was determined in a simulation involving the peroxide oxidation of small single-layer lecithin liposomes (4 mg/ml buffer) in the presence of a catalyst, namely methemoglobin (2.5 nM), at 40°C. Liposomes from lecithin-standard (Kharkov Plant) were prepared by means of the injection method [8] in 0.15 M NaCl with 0.01 M tris-HCl (pH 7.4). The peroxide oxidation process was followed by measuring oxygen consumption with a YSI Model 53 Biological Oxygen Monitor. AOA was expressed as the reduction in the initial rate of oxygen consumed (up to 35% of the original level) by the liposomes in the control experiments relative to that in the presence of the test substance:

$$AOA = V_0/V_i$$

In addition AOA was determined by comparing the level of β-carotene degradation at 50°C using a modified technique [9, 10]. The degradation process was monitored by measuring the reduction in extinction at 460 nm, a kinetic curve for the degradation of β-carotene being drawn from the data. The time taken for 30% conversion of β-carotene was taken as the induction period (τ). AOA was expressed as the increase in induction period in the presence of the test substance relative to that in the control experiments:

$$AOA = \tau/\tau_0$$

The ability of the test substances to incorporate into the lipid bilayer was judged from the relationship

$$I = \ln (F_0/F)$$

where $F_0$ and F are the fluorescence intensities of anthracene (in arbitrary units) in the absence and presence of the test dihydropyridines, respectively (see Table 4). Changes in the characteristic fluorescence of 4 μM of anthracene were registered as 380 nm (excitation at 340 nm) on a Hitachi 850 spectrofluorometer in phosphatidylcholine liposomes (0.5 mg/ml) in the presence of equimolar concentrations (5 μM) of the test dihydropyridines. As data existed on the fluorescence of dihydropyridine derivatives, specifically the coronary vasodilator riodipin 15, fluorescence spectra of the test substances were also recorded, in ethanol and in liposomes (see Table 3).

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