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Mono and dihydroxy coumarin derivatives: Copper chelation and reduction ability

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ABSTRACT

Due to the limited array of the currently available copper chelators, research of such compounds continues to be of clinical interest. Notably, *o*-dihydroxycoumarins have been previously shown to be potent iron chelators under neutral conditions. Within this study, the interaction of a series of natural coumarins and their synthetic analogs with copper has been evaluated in order to obtain structure-activity relationships under different pathophysiological pH conditions. Both competitive and non-competitive methods have been employed. Analysis of cupric ion reduction has also been performed.

Under mildly competitive conditions, cupric chelation was observed for *o*-dihydroxycoumarins, and partially for *o*-diacetoxycoumarin. Non-competitive studies showed that cuprous ions are not chelated at all and that the stoichiometries of the most active 6,7- and 7,8-dihydroxycoumarins to cupric ions ranged from 1:1 to 2:1 depending on pH and concentration. Interestingly, under highly competitive conditions, coumarins were not capable of chelating cupric ions, either. Reduction experiments have shown that 13 out of the 15 coumarins included in this study reduced cupric ions. However, significant differences depending on their structures were apparent in their potencies. *O*-dihydroxycoumarins were the most potent ones again.

Conclusion: O-dihydroxycoumarins are moderately active cupric ion chelators with potent copper reducing properties.

1. Introduction

Coumarins are 2H-benzopyran derivatives that constitute a diverse group of plant secondary metabolites ubiquitous in many plants from the families Apiaceae, Rutaceae, Fabaceae and Asteraceae [1]. The most simple natural coumarins are substituted by a hydroxy group (e.g. 7-hydroxycoumarin, umbelliferon), other simple natural hydroxy or methoxycoumarins are more highly oxygenated: 6,7-dihydroxycoumarin (aesculetin, 6,7-DHC), 7,8-dihydroxycoumarin (daphnetin, 7,8-DHC), 7-hydroxy-6-methoxycoumarin (scopoletin, HMC), 6,7-dimethoxycoumarin (6,7-DMC, scoparone) and their glycosides (e.g. 6-β-D-glucopyranosyloxy-7-hydroxycoumarin, aesculin). These simple compounds possess many potentially interesting biological properties including the ability to chelate metals and inhibit reactive oxygen and nitrogen species (ROS and RNS) – producing enzymes [2]. Some years ago, we demonstrated that *o*-dihydroxy-4-

Abbreviations: BCS, bathocuproinedisulphonic acid disodium salt; CCA, coumarin-3-carboxylic acid; 5,7-DAC, 5,7-diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin; 7,8-DAC, 7,8-diacetoxycoumarin-3-ethoxycarbonylmethyl-4-methylcoumarin; 5,7-DHC, 5,7-dihydroxy-4-methylcoumarin; 6,7-DHC, 6,7-dihydroxycoumarin; 7,8-DHC 1, 7,8-dihydroxycoumarin; 7,8-DHC 2, 7,8-dihydroxy-4-methylcoumarin; 7,8-DHC 3, 7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin; 7,8-DHC 4, 7,8-dihydroxy-3-ethoxycarbonylethyl-4-methylcoumarin; 6,7-DMC, 6,7-dimethoxycoumarin; 7,8-DMC 1, 7,8-dimethoxycoumarin; 7,8-DMC 2, 7,8-dimethoxy-3-ethoxycarbonylmethyl-4-methylcoumarin; HMC, 7-hydroxy-6-methoxycoumarin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

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methylcoumarins possessed good iron chelating properties [3]. Similar chelation was also suggested indirectly in the assays where iron chelation was not directly determined. However, inhibition of lipid peroxidation and hydroxyl radical production induced by ferric ions was observed [4]. This prompted us to investigate their ability to interact with copper. In contrast to iron, studies on copper chelation by coumarins are limited to a single report on the interaction of 6,7-DHC bound Cu^{2+} ions with catechol bisanion leading to a 1:1 complex [5]. Similarly, with a few exceptions [6,7], data on copper reduction are scarce. Indirect data involve an efficient inhibition of copper-catalysed oxidative modification of human LDL by *ortho*-dihydroxycoumarins. Copper chelation was, at least in part, responsible for this effect. Interestingly, 5,7-dihydroxycoumarin acted rather as a pro-oxidant via the promotion of the redox cycling of copper [8] and copper reduction could have been responsible for this effect. In summary, since copper chelation and reduction by coumarins has remained a virtually unexplored area, we decided to test a series of structurally related 15 coumarins using different competitive and non-competitive methods.

Iron and copper play essential roles in human physiology and, consequently, both excess and lack of the metals is associated with pathological states [9]. It is therefore not surprising that their chelation constitutes a possible therapeutic strategy. Ongoing research in this area is focused on the evaluation of several possible therapeutic indications starting from copper/iron excess conditions (hemochromatosis, Wilson's disease) through diabetes, neurodegenerative disorders to cancer [10–13]. As regards approved treatment modalities for iron and copper excess, there are three strong clinically used iron chelators (deferoxamine, deferasirox and deferipron) as compared to only one potent clinically used copper chelator (trientine). D-penicillamine should also be mentioned, but this substance seems to be a weak copper chelator regardless of its profound effect on copper excretion [14,15]. The most modern approach towards copper excess (Wilson's disease) treatment is based on the use of tetrathiomolybdate. This drug, however, has not been approved by FDA, and its mechanism of action is probably based on the formation of zinc-tetrathiomolybdate-protein complex [16]. As stated above, research of copper or metal chelators has been directed towards their potential impact on some neurodegenerative disorders, in particular on Alzheimer disease. Mostly the drugs derived from 8-hydroxyquinoline have been tested [17,18]. But, surprisingly, clioquinol was shown to act rather as a metal redistributor than a copper chelator [19,20]. Thus, given the very limited amount of the current knowledge, research of novel copper chelators is highly desirable. However, considering the huge therapeutic potential, different properties are clearly needed for various indications. For example, to increase metal elimination under metal overload conditions, strong chelators are needed, while complex-forming compounds with redox-cycling properties are likely to be more suitable for tumor treatment [11,21]. Hence, both copper chelation and reduction have been analysed within this study. In addition, since there are (patho) physiological differences in pH, e.g. acidic pH in the gastrointestinal tract, tumor cells or during ischaemia, all the assays were performed in acidic to neutral conditions [22–24].

2. Materials and methods

2.1. Reagents

Synthetic coumarins 5,7-dihydroxy-4-methylcoumarin (5,7-DHC), 7,8-dihydroxy-4-methylcoumarin (7,8-DHC 2), 7,8-dihydroxy-3-ethoxycarbonylmethyl-4-methylcoumarin (7,8-DHC 3), 7,8-dihydroxy-3-ethoxycarbonylethyl-4-methylcoumarin (7,8-DHC 4), 7,8-diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (7,8-DAC), 7,8-dimethoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (7,8-DMC 2) and 5,7-diacetoxy-3-ethoxycarbonylethyl-4-methylcoumarin (5,7-DAC) were synthesized using literature procedures [25–27] at the Department of Chemistry, University of Delhi, India. Their purity was ≥95%. 7-Hydroxycoumarin, 6,7-dihydroxycoumarin (6,7-DHC), 6,7-dimethoxycoumarin (6,7-DMC), 7,8-dihydroxycoumarin (7,8-DHC 1), 7,8-dimethoxycoumarin (7,8-DMC 1), HMC, aesculin and coumarin-3-carboxylic acid (CCA) were purchased from Sigma-Aldrich (Germany) as well as cupric sulphate pentahydrate, cuprous chloride, hydroxylamine hydrochloride, disodium bathocuproinedisulfonate (BCS) and DMSO. Methanol was purchased from J.T. Baker (Avantor Performance Materials, Inc., USA). Ultrapure water (Milli-Q RG, Merck Millipore, Massachusetts, USA) was used throughout this study. A stock solution of cuprous ions (5 mM) was prepared by the dissolution of cuprous chloride (CuCl) in an aqueous solution of 0.1 M HCl and 1 M NaCl. Working solutions were prepared using distilled water. Cupric ions 5 mM (cupric sulfate pentahydrate, $CuSO_4{\cdot}5H_2O$) were dissolved directly in distilled water.

Acetate buffers (15 mM of sodium acetate salt and 27,3 and 2.7 mM of acetic acid, respectively) were used for the two lower pH values (pH 4.5, 5.5) whereas HEPES buffers (15 mM of sodium HEPES and 71,7 and 14,3 mM of HEPES, respectively) for pH 6.8 and 7.5.

2.2. Methods

2.2.1. Copper competitive chelation assays

The hematoxylin method was used according to the previously published procedure [14]. Briefly: DMSO solutions of a coumarin (50 μL) were mixed with the same volume of 250 μM solution of cupric ions in a buffer of pH 5.5–7.5 for 2 min in a 96-well microplate. Thereafter, hematoxylin (50 μL, 250 μM) or DMSO (blank) was added and absorbance was measured at 610 nm (pH 7.5), 590 nm (pH 6.8) and 595 nm (pH 5.5) after 3 min and 7 min. Bathocuproine assay (BCS assay) was also performed according to the procedure published previously by us [14]. Similar to the previous method, DMSO solutions of the coumarins in different concentrations were mixed with either cupric or cuprous ions (50 μL of both) in a buffer (pH 4.5–7.5). In the case of cuprous ions, hydroxylamine (50 μL, 10 mM) was present in the mixture in order to inhibit copper oxidation while in the case of cupric ions, hydroxylamine was added after mixing in order to reduce non-chelated cupric ions. In the last step, BCS (50 μL, 5 mM) or water (blank) was added, and absorbance was measured at 484 nm immediately and after 5 min. In both chelation assays, the final concentration of cupric or cuprous ions was 50 μM while the final concentration of coumarin ranged from 500 nM up to 5 mM depending on the activity.

2.2.2. Copper reduction assay

Reduction experiments were performed in the same way as the BCS assay except that the reducing agent hydroxylamine was not added to the samples, but used only as the positive control (100% reduction). The final concentration of cupric or cuprous ions was again 50 μM, while the final concentration of coumarin ranged from 100 nM up to 5 mM depending on the activity. Absorbance was measured again immediately at 484 nm and after 5 min.

All the above-mentioned experiments were performed in 96-well microplates, and absorbance was measured using the Synergy HT Multi-Detection Microplate Reader spectrophotometer (BioTec Instruments, Inc., Winooski, Vermont, USA). Every measurement was done at least in duplicate, and experiments on each compound were repeated with two independent stock solutions as a minimum.

2.2.3. Copper non-competitive assays – determination of the complex stoichiometry

The assessment of the stoichiometry was performed at four (patho) physiologically relevant pH values (4.5, 5.5, 6.8 and 7.5) using the same buffers as above with the Helios Gamma spectrophotometer equipped with the VisionLite 2.2 software (ThermoFisher Scientific Inc., USA). Firstly, absorption spectra ranging from 220 to 800 nm were scanned, and the wavelength(s) of the absorption maximum(a) of a tested substance was/were determined under all pH conditions. The