Allura Red, the Artificial Azo Dye, Inhibits Esterase Activity of Carbonic Anhydrase II: A Preliminary Study on the Food Safety in Term of Enzyme Inhibition

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Allura red (AR) is a widely used colorant in food industry, but there is debate on its potential security risk. In this study, *in vitro* inhibitory properties of the dye against carbonic anhydrase (CA) were evaluated. The esterase activity of purified CA decreased in the presence of AR, in a dose-dependent manner. Regarding literature review and observed results, this preliminary study may provide new horizons in safety of AR and the other dye additives.

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Introduction

Human carbonic anhydrase II (hCAII, EC 4.2.1.1) is a metalloenzyme that catalyzes the reversible hydration of CO_2 to HCO_3^-. This single domain protein is composed of 260 amino acids with a central stranded sheet. The catalytic zinc is coordinated by the imidazole moieties of three histidines located deep in a conical amphiphilic binding pocket [1] (Fig. 1).

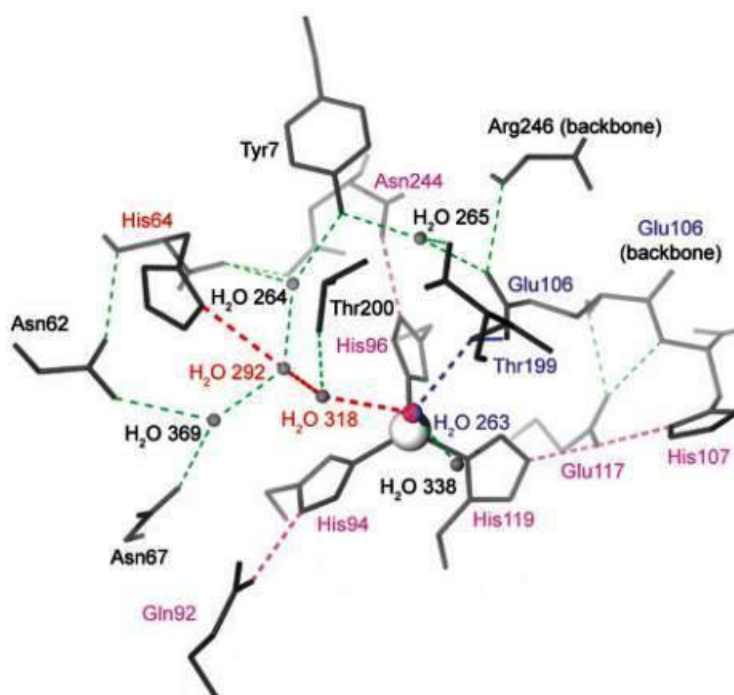

Fig. 1. The active site of HCA II in a stick diagram taken from ref [2]. The zinc ion and water molecules are shown as gray spheres. All hydrogen bonds involving solvent molecules are represented in dashed lines. For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.

In the ligand-free state, water molecules fill the active site. One of them, presumably present as OH-, occupies the fourth coordination site at zinc. It is also hydrogen-bonded to Thr[199], which in turn is assumed to form a hydrogen bond to the substrate CO_2. An additional water, H_2O^{338} (the so-called "deep water"), is found in the ligand-free state and replaced in the enzymatic reaction by CO_2 (see Fig. 2) [1].

For a long time, it was believed that carbonic anhydrase exhibited absolute specificity. However, in the 1960s it was discovered that the enzyme also catalyzes hydration of various aldehydes as well as hydrolysis of esters [1 and references therein]. According to the vital physiological roles of CAs, it seems plausible that modulation of CA activity to normal levels either by inhibition or activation offers interesting therapeutic options [3]. Blockade of CA activity in local tissues may therefore increase tissue CO_2 concentrations and/or lower tissue pH, resulting in vascular dilation and increased blood flow. Deficiency of hCA is the primary defect in the syndrome of osteopetrosis, renal tubular acidosis, and cerebral calcification [1 and references therein]. CA inhibitors are widely used clinically for the treatment or prevention of a multitude of diseases (Fig. 2).

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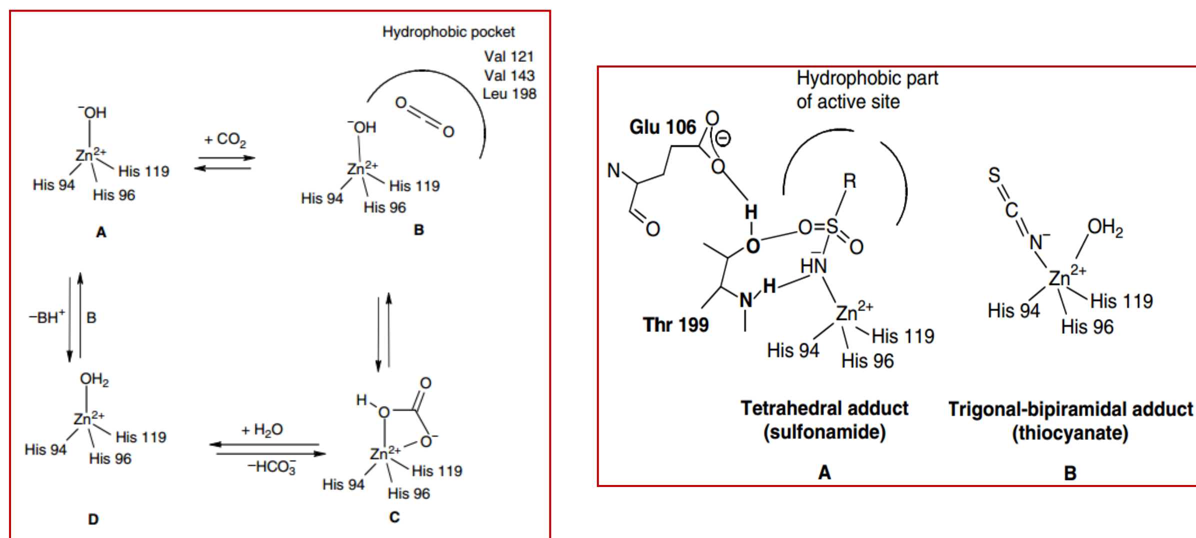

Fig. 2. (***Left***) Schematic representation of the catalytic mechanism for the CA-catalyzed CO_2 hydration. The hypothesized hydrophobic pocket for the binding of substrates is shown schematically at Step B.. (***Right***) CA inhibition mechanism by sulfonamide (A) and anionic (B) inhibitors. In the case of sulfonamides, in addition to the Zn(II) coordination, an extended network of hydrogen bonds ensues, involving residues Thr 199 and Glu 106, whereas the organic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. For anionic inhibitors such as thiocyanate (B), the interactions between inhibitor and enzyme are much simpler.

Regarding vital physiological roles of CAs, it appears that inhibition or activation of these lyase proteins by natural compounds and/or food additives lead to outstanding changes on the normal metabolism. Food dyes are largely used in food industry to improve the appearance of foods with purpose of attracting consumers. Compared with natural dyes, synthetic colorants have more applications because of their high stabilities, color uniformity, low microbiological contamination and relatively lower production costs [4]. Specifically they are used to offset color loss due to exposure to light/air/temperature extremes, moisture and storage conditions; and to provide color to colorless items. Processed foods such as cookies, candy, cakes, sports drinks, margarine and cheeses tend to contain the highest amount of color additives [4, 5]. Coloring is also used widely in food products marketed to children such as candy, soft drinks and breakfast cereals to help increase the products' visual appeal on shelf, excite the senses and bump up the perceived fun factor. While natural colorants such as vegetable leaves, pomegranate and saffron are generally a safer alternative, today the majority of modern coloring is derived from coal tar or petroleum or created by chemical synthesis by food manufacturers because it's more economical. However, some synthetic colorants are harmful to human body, particularly if they are excessively consumed [4, 5]. Therefore, like many other food additives, control in the dosage of dyes is of considerable importance in food industry [6].

Allura red, (E129) is an artificial red azo dye that goes by several names including: Food Red 17, CI 16035 and FD&C Red no.40. It is most often used in soft drinks, cosmetics, candy, chewing gum and condiments. This additive was introduced in the early 1980s to replace Amaranth, a dye that was banned by the U.S. Food and Drug Administration during that time. Allura red (AR) belongs to the water-soluble, monoazo class of synthetic food pigment, with excellent stabilities in many food products such as candy coating, ice cream, drinks and confections (Fig. 3). However, it has been reported that AR exhibited toxicity in rats, and

was decomposed to produce some toxic, mutagenic and carcinogenic aromatic amines by the intestinal microflora [6]. It was found that AR can induce DNA damage in the colon of the mice [7]. Due to the existence of potential security risks, AR is not recommended for consumption by children in Europe [8]. Allura red is currently banned in Denmark, Belgium, France, Germany, Switzerland, sweden, Austria and Norway [9]. Taking these reports into consideration and despite huge studies performed on the safety/possible toxicity of AR [8], there is yet important conflicting issues remained to be resolved and more accurate and comprehensive safety evaluation of AR is indispensable. The possible inhibitory/activatory effects of AR on the vital/regulatory enzymes may be considered as an important control [6]. Some functional groups in AR are similar to sulfonamide moieties of potent CA inhibitors (Fig. 3), so there is this possibility that AR inhibit CA activity. In the current study, we report a preliminary study on CA II inhibition by AR and then discuss more on AR safety regarding its potential for CA inhibition.

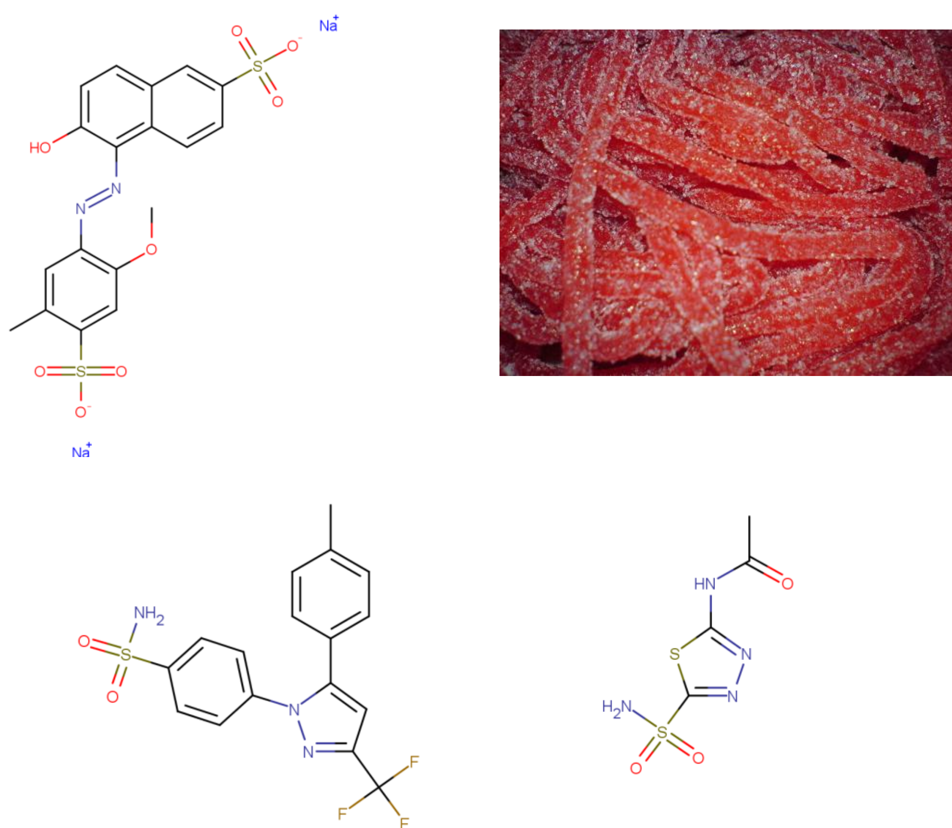

Fig. 3. Chemical structures of Allura Red AC (Top, Left), CA II inhibitors; celecoxib (Bottom, Left) and acetazolamide (Bottom, Right). Allura Red AC in confectionery (Top, Right).

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Materials and Methods

Protein purification

Human CA II was purified from human erythrocytes according to the method described by Nyman and the enzyme purity was confirmed by SDS-PAGE [10].

Carbonic anhydrase assay

Activity of hCA II was assayed based on *p*NPA esterase activity of the enzyme [10] in the absence and the presence of the different concentrations of dye. The catalytic reaction was monitored at 400 nm (due to liberation of *p*-nitrophenol as the hydrolysis product) using a PerkinElmer Lambda 25 UV-Vis spectrophotometer in quartz cells with 1 cm pathlength. In order to determine the mode of inhibition and the inhibitory constant, hCA II activity was assayed in a variety of *p*NPA concentrations in the presence of increasing concentrations of AR using appropriate blank. The initial velocity, V_0, was determined as the slope of the absorbance changes at 400 nm during the linear phase of the catalytic reaction. The dye and CA II were allowed to reach equilibrium for 1 min in the assay solution before adding *p*NPA as the substrate. All the experiments were done in triplicate and results were expressed as mean±SD with $P<0.05$. IC50, K_m and V_{max} values were also calculated by GraphPad Prism v5.

Results

Determination of the mode of inhibition

Previous studies have shown that the CA inhibitor, celecoxib, is able to inhibit carbonic anhydrase activity with the IC_{50} of nM both for lyase and esterase activities [10]. Based on the literature, the elements of both lyase and esterase active sites of CA are the same so that enzymatic hydrolysis of esters appears to be either controlled by the same amino acid side chains and the same mechanism [1]. Thus we evaluated CA inhibition by AR based on esterase activity of the enzyme.

However, there is also not any report on ascertaining the mode of inhibition of AR. As indicated in Fig. 4, AR showed weak CA II inhibitory property, so that ~ 99% activity was suppressed at 900 μM concentration of AR (IC50=565±20.91). The double reciprocal, Lineweaver-Burk, plot (Fig. 5) is the most straightforward means of diagnosing inhibitor modality. A plot of $1/V_0$ versus 1/[S], a Lineweaver- Burk plot, yields (in most cases) a straight line with an intercept of $1/V_{max}$ and a slpoe of K_m/V_{max}. Overlaying the double reciprocal lines for an enzyme reaction carried out at several fixed inhibitor concentrations will yield a pattern of lines that is characteristic of a particular inhibitor type. The results of Lineweaver-Burk plot (Fig. 5) revealed that AR inhibits the esterase activity of hCA II in a competitive manner. So, it can be concluded that the substrate (*p*NPA) and the inhibitor (AR) compete for binding to the same site (active site).

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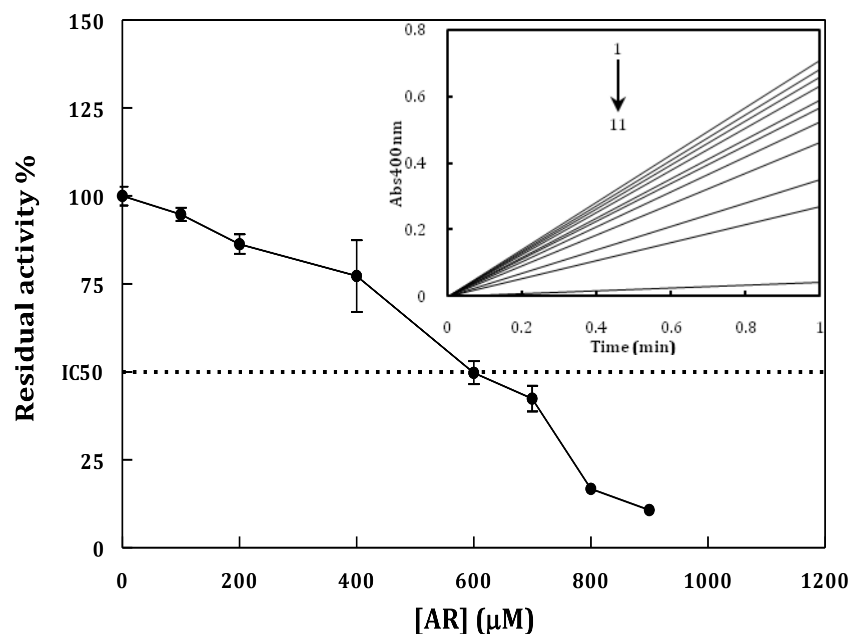

Fig. 4. Residual esterase activity of HCA II in the presence of different concentrations of AR. Production of *p*-nitrophenol was monitored by absorbance at 400 nm in the absence (1) and the presence of 100-900 μM of AR (2-11). Data shown are representative example of three independent experiments and standard deviations were almost within 5% of the experimental values.

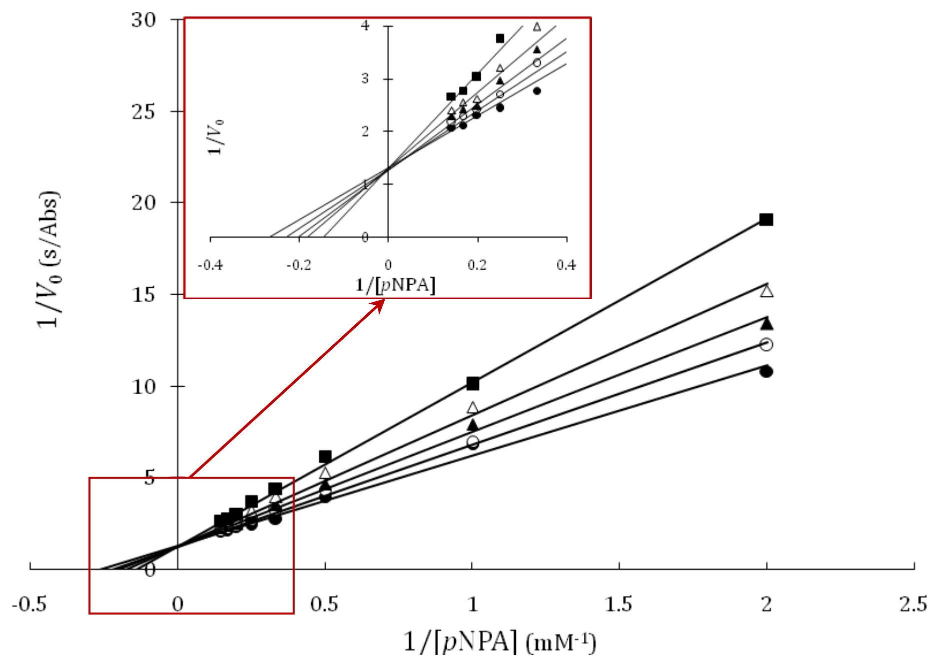

Fig. 5. The Lineweaver-Burk plot of HCA II esterase activity in the absence (●) and the presence of 50 (○), 100 (▲), 200 (△) and 300 μM of AR (■). Data shown are representative example of three independent experiments and standard deviations were approximately within 5% of the experimental values.

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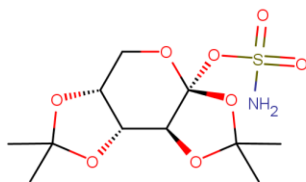
Two main classes of carbonic anhydrase inhibitors (CAIs) are known: the metal-complexing anions and the un-substituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the nonprotein zinc ligand or add to the metal coordination sphere (Fig. 2) [1, 11, 12]. Sulfonamides, the most important CAIs, bind in a tetrahedral geometry of the Zn(II) ion, in a deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II) and an extended network of hydrogen bonds involving the residues Thr 199 and Glu 106, also participating in anchoring the inhibitor molecule to the metal ion. The aromatic/heterocyclic part of the inhibitor (R) interacts with hydrophilic and hydrophobic residues of the cavity. Anions might bind either in tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts, such as the thiocyanate adduct shown in Figs. 2 and 3. Sulfamides ($H_2NSO_2NH_2$), and sulfamates ($-OSO_2NH_2$), are also potent inhibitors of CAs (Fig. 6).



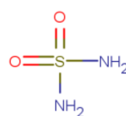


Fig. 6. Chemical structures of two sulfamate (topiramate) and sulfamide inhibitors of CAs.

Despite important similarities in the binding of these two inhibitors to the enzyme to that of aromatic/heterocyclic sulfonamides of the type RSO_2NH_2, the absence of a $C–SO_2NH_2$ bond in sulfamide/sulfamate leads to a different hydrogen bond network in the neighborhood of the catalytical Zn(II) ion, which has been shown to be useful for the design of more potent CA inhibitors as drugs, possessing zinc-binding functions different from those of classical sulfonamides [11, 12].

In the current work, we showed that the azo dye, allura red (AR), inhibited esterase activity of CA II. Since AR contains $R\text{-}SO_3^-$ anion as well as apolar aromatic rings, there is the possibility that the azo dye both mimics interactions of anionic inhibitors with zinc ion, and also, similar to the aromatic sulfonamide, celecoxib, interacts with hydrophobic residues of the enzyme cavity, through its aromatic moieties. More importantly, it has been postulated that reduction of azo by gut flora of the dye will yield the two components of the parent compound: 2-methoxy-5-methyl-aniline-4-sulfonic acid (cresidine-4-sulfonic acid) and 1-amino-2-naphthol-6-sulfonic acid (Fig. 7)[a]. Although it appears that negligible quantities of intact Allura Red are absorbed and excreted in the urine, and that the major portion of the color is excreted as metabolites in the feces, there is this possibility that significant amounts of reduced metabolites enter blood circulation and affect extracellular and intracellular enzyme activities. Additionally, their inhibitory activity may be different compared to that of parent compound due to smaller molecular size and more availability for enzyme active sites.

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Fig. 7. Chemical structures of two components of AR: 2-methoxy-5-methyl-aniline-4-sulfonic acid (cresidine-4-sulfonic acid) and 1-amino-2-naphthol-6-sulfonic acid.

Although vegetable-based dyes has been recently used to create brightly colored foods; as stated earlier, artificial color additives are extensively employed to enhance/correct natural colors and "provide a colorful identity to foods that would otherwise be virtually colorless," as well as compensating for natural color loss during storage and providing a way to quickly identify pharmaceuticals and dietary supplements. In addition to AR, there is this possibility that many other azo dyes with similar molecular structures (Fig. 8) show the same/or more potent inhibitory activities on CA enzyme. Sunset yellow, for instance, is used in food, cosmetics, and drugs. It is generally used in candy, desserts, snacks, sauces, and preserved fruits. It is often used in conjunction with amaranth, to produce a brown coloring in both chocolates and caramel. Products containing tartrazine, also, commonly include processed commercial foods that have an artificial yellow or green color, or that consumers expect to be brown or creamy looking. The above expected effects may appear more important, due to large consumption rate of these dyes. Food dye consumption per person has increased fivefold in the United States since 1955, with three dyes—Red 40 (AR), Yellow 5 (tartrazine), and Yellow 6 (sunset yellow)—accounting for 90% of the dyes used in foods [13]. Evaluation of possible CA-esterase inhibitory properties of these known dyes as well as their metabolites are in process, in our Lab.

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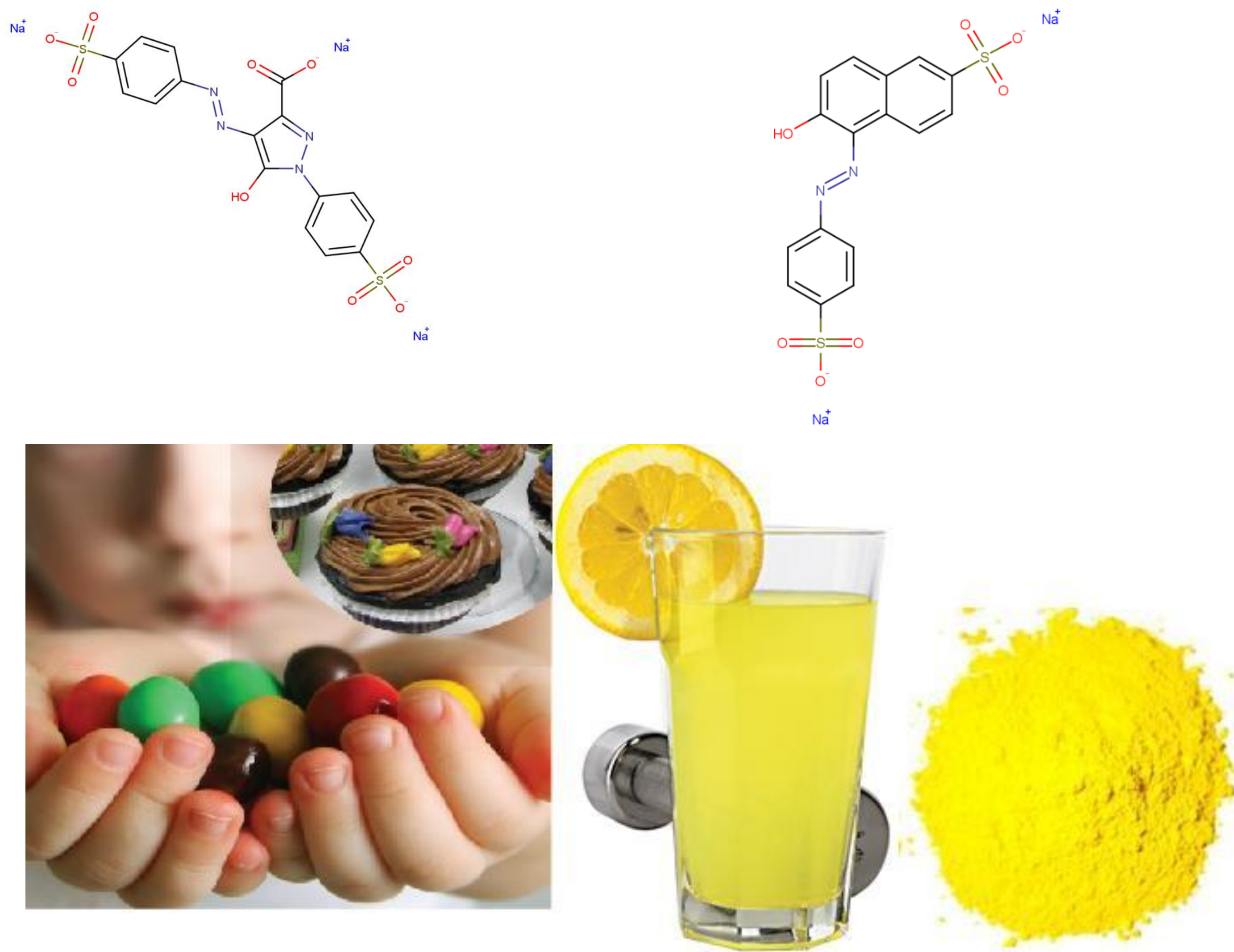

Fig. 8. *Top,* chemical structures of sunset yellow (right) and tartrazine (left). *Bottom*, left; Artificial dyes are not the only way to create brightly colored foods; many countries use vegetable-based dyes (see sample wares in the inset) to achieve the same effect [14]. Right; sunset yellow dye; ready to drink/and as pure powder forms. For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.

Conclusion

These days AR has an acceptable daily intake (ADI) of 7 mg/kg bw/day (210 mg for a 30-kg child)[13]. Moreover, several companies produce the equivalent of about 25 mg of the dye per person per day, with many children probably averaging several times as much. Based on the results of this preliminary study, since both intact Azo-dyes and their degradation products can be considered as inhibitor of CA esterase activity, further study on their possible effects on lyase activity of the enzyme is of great importance, especially due to large consumption rate of these artificial colorants. It appears that enzyme inhibition/activation will be considered, more

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seriously, as a new opened dimension in food safety.

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Conflict of interest

Authors do not have any conflict of interest with the commercial identities mentioned in this article.

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