Inhibition of Protein Tyrosine Phosphatase 1B by Aurintricarboxylic Acid and Methylenedisalicylic Acid; Polymer *versus* Monomer

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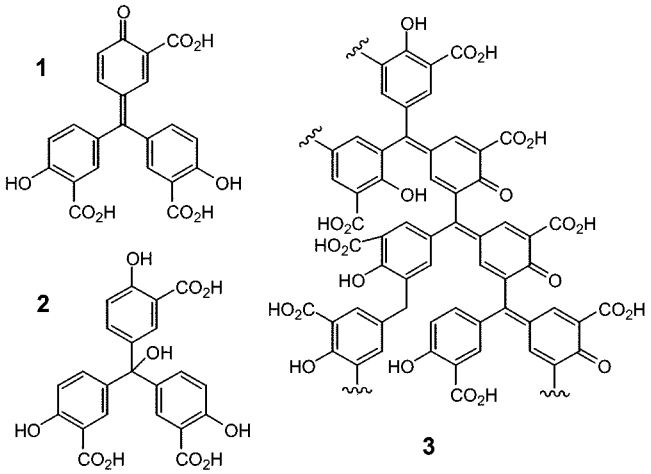
Aurintricarboxylic acid (ATA) is a substance that has been known to prevent apoptotic cell death in numerous cell types.[1-4] ATA is also known to exhibit inhibitory activity against a broad range of enzymes and this property has been utilized in many biological experiments.[5-7] ATA is represented in many of the literature as a chemical containing three salicylic acid moieties (**1**, **2**).[8] Commercial ATA, however, contains significant amounts of polymeric materials schematically represented as **3**.[9] In general, ATA is prepared by condensation of salicylic acid with formaldehyde and the branching reaction results in the formation of polymers of molecular weights up to several thousands Dalton.[9-12]



Recently, it was found that ATA is a potent inhibitor of protein tyrosine phosphatases (PTPases).[13] This observation raised our interest because ATA has been known to prevent cell death in several cell types including PC12 cells which undergo apoptosis in the absence of serum and growth factor.[1,2] Because of its polyanionic character, ATA has been considered membrane impermeable and the antiapoptotic effect of ATA has been explained by its extracellular binding to certain growth factor receptor kinases in MDA-231 cells.[14] Contradictory to this premise, we recently found that ATA translocates across the plasma membrane in PC12 cells and proposed that the antiapoptotic effect of ATA might be due to the inhibition of endogenous PTPase(s).[15]

In this study, we examined whether the *in vitro* inhibitory activity of ATA against PTPases resides in the monomer or high molecular weight components. Not to mention commercial ATA, the ATA sample synthesized according to the method[8] (method A, Scheme 1) previously reported to produce monomer was also found to contain polymeric materials as described below. Therefore, monomeric component of ATA (**2**) was prepared absolutely free of polymer. Also synthesized in a pure form was methylenedisalicylic acid (MDSA), one of the low molecular weight components formed in the conventional preparation of ATA. Commercial MDSA was also proved to contain polymeric substances. The inhibitory potency of ATA and MDSA synthesized in a polymer-free form was evaluated against human protein tyrosine phosphatase 1B (PTP1B).

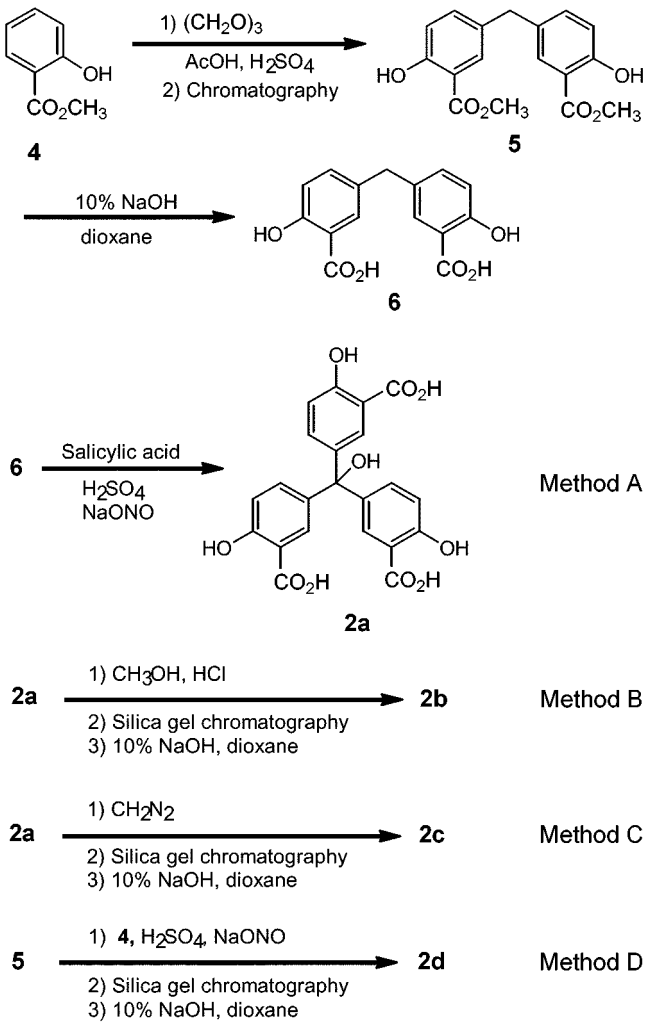
Dimethyl methylenedisalicylate (**5**) was prepared by the reaction of methyl salicylate and trioxane according to the reported procedure (Scheme 1).[16] It was purified by column chromatography to remove any unwanted by-products containing more than two salicylic acid moieties and then hydrolyzed to obtain polymer-free MDSA (**6**). Carbinols **2a-2d** were synthesized from MDSA or its dimethyl ester in four independent routes. They are basically the same compound containing different amounts of polymeric by-products. In methods B and C, **2a** was esterified, purified and hydrolyzed to obtain **2b** and **2c**. Another strategy (method D) was to condense **4** and **5**. The resulting triester was column-purified and hydrolyzed to obtain **2d**.

We evaluated the inhibitory potency of the synthesized carbinol samples **2a-2d**. Samples **2a-2d** (IC_{50} = 18-120 μM) were significantly less potent compared to the commercial ATA (IC_{50} = 0.5 μM) indicating that high molecular weight components of commercial ATA are more effective inhibitors of PTP1B. Among **2a-2d**, **2a** was > 4-fold more potent compared to **2b-2d**. This is probably due to the differences in the contents of the contaminating polymeric substances, because the polymeric by-products were removed during the syntheses of **2b-2d** but not for synthesis of **2a**. Even though method A was developed previously to obtain monomeric ATA, this method was not absolutely free from polymer formation and the contaminants played major role in the inhibition of PTP1B. Considering that the IC_{50} values of commercial ATA and **2d** were 0.5 μM versus 120 μM, a 240-fold difference, sample **2d** mixed with just a few percent of commercial ATA would exhibit IC_{50} value much lower than 120 μM. Commercial sample of MDSA was also

Table 1. Inhibition of PTP1B by methylenedisalicylic acid (**6**) and 3,3',3"Tricarboxy-4,4',4"-trihydroxytriphenylcarbinol (**2a-2d**) samples prepared in four different strategies

	Commercial **2**[b]	**2a**[c]	**2b**[c]	**2c**[c]	**2d**[c]	Commercial **6**[b]	**6**[c]
IC_{50} (μM)[a]	0.50 ± 0.14	18 ± 2	89 ± 18	93 ± 8	120 ± 15	20 ± 11	3600 ± 790

[a]IC_{50} values were obtained using 10 mM *p*-nitrophenyl phosphate as a substrate.[17] Steady-state kinetic experiments revealed that monomeric sample **2d** and commercial ATA are both competitive inhibitors of PTP1B (Data not shown). [b]Purchased from Sigma. [c]Synthesized as in Scheme 1. Compounds **2a-2d** are compounds with identical chemical structure prepared in different routes and, therefore, they contain different amounts of polymeric materials as minor impurities.



Scheme 1. Synthetic strategies for the syntheses of methylenedisalicylic acid (**6**) and 3,3',3"-tricarboxy-4,4',4"-trihydroxytriphenylcarbinol (**2a-2d**) in four different routes.

recognized to contain polymeric materials that might deceive experimental results. As shown in Table 1, commercial sample exhibited IC_{50} of 20 μM, a 180-fold lower value than that of polymer-free MDSA.

ATA is listed as an apoptosis inhibitor in catalogs of commercial providers including Oncogene Research Products (Darmstadt, Germany). To the best of our knowledge, most, if not all, of the experiments utilizing ATA as an apoptosis blocker have used commercial ATA.[1,2,4] The latter, however, is not a homogeneous substance. ATA synthesized according to a commonly used procedure had previously been fractionated to a mixture of more than twenty substances in a broad molecular weight range.[9-12] Even though each of the components are not completely characterized, the structure of the polymeric substances are generally described as **3**.[9] It is not evident at this stage whether the anti-apoptotic effect of ATA resides in the monomeric form or in high molecular weight components. However, if the effect of ATA is directly related with its inhibitory activity against PTPases as recently proposed by us,[15] polymeric components are likely to be more active ingredients.

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