Separation of Biologically Active Lipids from Red Wine

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A number of lines of evidence suggest that red wine exerts a protective effect against coronary heart disease, but the nature of the protective compounds is unclear and the mechanism is incompletely understood. In this study, total lipids of a Greek red wine were separated into neutral and polar lipids. Polar lipids were further separated into glyco- and phospholipids, which were fractionated by HPLC. Each lipid fraction was tested in vitro for its ability to inhibit platelet-activating factor (PAF) and thrombin-induced washed rabbit platelet aggregation and/or to cause platelet aggregation. A significant number of glyco- and phospholipids that exerted the above biological activities were detected. Structural data of an active phosphoglycolipid are also provided. *trans*-Resveratrol demonstrated also a dose-dependent inhibition of PAF-induced platelet aggregation along with the already reported inhibitory activity against thrombin and adenosine-5′-diphosphate. Because it has already been reported that PAF is involved in atheromatosis generation, the existence of PAF inhibitors in red wine may contribute to the protective role of red wine against atherosclerosis.

Keywords: *Red wine; lipid analysis; platelet-activating factor; resveratrol; Mediterranean diet*

INTRODUCTION

Atherosclerosis, the major cause of mortality and morbidity in developed countries, is characterized at its early stage by macrophage cholesterol accumulation and foam cell formation. The cholesterol accumulating in these cells is derived mainly from plasma low-density lipoprotein (LDL) that has undergone oxidative modification (Aviram et al., 1996; Berliner et al., 1995).

As it has been published (Gurr, 1992) as well as reported in the Symposium on Nutrition and Atherosclerosis held in October 1994, a satellite of the Xth International Symposium on Atherosclerosis in Montreal, fat restriction does not result in proportional reduction of the risk factors for coronary heart disease. On the other hand, there is strong evidence from epidemiological data that other dietary components such as antioxidants and especially vitamin E play an important role in preventing coronary heart disease (Chan, 1998).

Also, the "response-to-injury" hypothesis (Chan, 1998) emphasizes the similarity of the inflammatory response to atherosclerotic lesions, implicating endothelial injury as a primary determinant for cardiovascular disease. 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF; Demopoulos et al., 1979) is mediator in inflammation, immune disorders, and ischemic diseases (Koltai et al., 1991) and is synthesized, released, and metabolized by inflammatory cells, such as macrophages, polymorphonuclear leukocytes (PMNL), and platelets as well as by endothelial cells. It has also been reported that PAF may be involved in atherogenesis in several ways: (i) its metabolic path is related to lipid metabolism, which profoundly changes in atherogenesis; (ii) it is a strong inflammatory mediator; and (iii) it is a potent vasoactive mediator that induces abnormal vascular reactions (Koltai and Braquet, 1992).

We recently reported the mechanism by which PAF is implicated in atheromatosis generation (Antonopoulou and Demopoulos, 1997) based on a previous detection of PAF, which is produced during LDL oxidation (Liapikos et al., 1994) and causes in situ inflammation. Briefly, PAF action results in endothelium destruction and gap formation, as well as in the proliferation of smooth muscle cells in which several cells such as foam cells and platelets adhere. In addition, other blood constituents such as lipids also adhere in the above complex, generating the atheroma plaque. This mechanism is also supported by in vivo experiments performed by other investigators (Feliste et al., 1989; Lehr et al., 1992), in which atheromatosis generation was not detected in the experimental animals fed cholesterol-rich food along with specific inhibitors of PAF.

The above findings show that specific PAF inhibitors may protect against atheromatosis generation. On the basis of this assumption, we detected lipid compounds that inhibit PAF actions in Mediterranean foods, namely, olive oil (Koussissis et al., 1993), honey and wax (Koussissis et al., 1994), milk and yogurt (Antonopoulou et al., 1996), fishes (Rementzis et al., 1997), and plants (Antonopoulou et al., 1996).

Wine is an important component in Mediterranean dietary traditions: it is claimed to contribute to the explanation of the "French paradox" (Frankel et al., 1993; Renaud and De Lorgeril, 1992). Earlier investigations have concurred in the conclusion that not only wine in general but red wine specifically is the most potent alcoholic beverage conferring protection against

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atherosclerosis (Serafini et al., 1998). Attention has been directed to red wine constituents to define their protective role as antiatherogenic agents. Until now, the potential cardioprotective effects of red wine are proposed to be mainly related to the antioxidant activity of nonalcoholic phenolic components. Phenolic flavonoids such as resveratrol and quercetin resulted in LDL enrichment (Aviram and Fuhrman, 1998) and inhibition of LDL oxidation in vitro (Kerry and Abbey, 1997). Additionally, red wine phenolics are reported to act upon platelets either by causing serotonin secretion, in other words, platelet activation (Rotondo et al., 1996; Pattichis et al., 1993), or by inhibiting platelet aggregation induced by thrombin and adenosine 5′-diphosphate (ADP) (Pace-Asciak et al., 1995, 1996; Subbaramaiah et al., 1998). Also, red wine raises plasma HDL cholesterol (Goldberg et al., 1996). It should be mentioned that resveratrol has been reported to show cancer chemopreventive activity, too (Jang et al., 1997).

Although a lot of work has been done in the identification of the phenolic constituents of red wine, little information exists on the lipid composition of both white and red wines, which is reported to be influenced by the lipid component in yeast (Zherebin et al., 1981; Portnova, 1978).

The above data, although significant, are controversial because experiments with purified phenolics produce less significant results than red wine and in some cases white and red wine appear to have comparable results, even though the only consistent difference between them is the phenolic content, being 20 times higher in red wine (Frankel et al., 1995). These results suggest that there are compounds present in red wine other than the phenolic ones that should be responsible for the protective role of red wine.

Our previously reported mechanism of atheromatosis generation may offer a new insight into pathological events in which PAF may be involved. In this work, we investigate the existence of PAF and thrombin inhibitors in lipids of red wine as well as the inhibitory activity of the most studied phenolic component of red wine.

EXPERIMENTAL PROCEDURES

Apparatus. HPLC was performed on a dual pump Jasco (Tokyo, Japan) model 880-PU HPLC, supplied with a 330 μL loop Rheodyne (P/N 7125-047) injector. A Jasco model 875 UV spectrophotometer was used as detector (205−210 nm). The spectrophotometer is connected to a Hewlett-Packard (Avondale, PA) model HP-3396A integrator−plotter. The following columns were used: a cation exchange column, SS 10 μm Partisil 25 cm × 4.6 mm i.d., PXS 10/25 SCX from Whatman (Clifton, NJ); and an adsorption column, silica 25 cm × 4.6 mm i.d., from Hichrom H5 (Reading, Berkshire, U.K.). The flow rate was 1 mL/min. The PAF-induced aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer coupled to an Omniscribe recorder (Houston, TX). The biologically active purified lipid fraction was subjected to mass spectrometry analysis. The electrospray mass spectrometry (ESMS) was recorded on a Fisons VG Quattro instrument with a VG Biotech electrospray source, having a hexapole lens. Nitrogen of 99.99% purity was used as the nebulizing and bath gas at flows of 20 and 150 $dm^3\ min^{-1}$, respectively. The sample was injected in the flow of solvent (10 μL/min) of a Varian 9012 solvent delivery system, via a Fisons interface with a Rheodyne 7125 injector. The capillary voltage was optimum at ∼3.30 kV for positive ions. The high-voltage lens potential was kept at 0.56 kV. The focus and skimmer lens voltages were 40 and 45 V, respectively, in the majority of the measurements, as these values produced the highest peak intensities and minimum fragmentation. HPLC grade methanol/water (70:30, v/v) 0.01 M in ammonium acetate was used as solvent.

Reagents. All reagents and chemicals were of analytical grade supplied by Merck (Darmstadt, Germany). Resveratrol was purchased from Sigma (St. Louis, MO). Red wine, Cabernet Sauvignon (12^0), 1995, was kindly offered by Domaine Hatzimichalis. High-performance liquid chromatography (HPLC) solvents were purchased from Ruthburn (Walkerburn, Peebleshire, U.K.). Lipid standards of HPLC grade were obtained from Supelco (Bellefonte, PA). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (Demopoulos et al., 1979). Chromatographic material used for thin-layer chromatography (TLC) was silica gel G-60 (Merck).

Procedure. Total lipids were extracted from 2 L of red wine according to the Bligh−Dyer method (Bligh and Dyer, 1959). Pigment contamination from the above sample was removed by two successive preparative TLC runs (Demopoulos et al., 1996). The fractions of polar and neutral lipids were pooled together, and the fraction of total lipids free from pigments was obtained. The lipid fraction was separated in neutral and polar lipids by countercurrent distribution (Galanos and Kapoulas, 1962). Polar lipids were further fractionated on HPLC (Andrikopoulos et al., 1986). Briefly, polar lipids were separated on a cation exchange HPLC column 10 μm using an isocratic elution system consisting of acetonitrile 60% and methanol/water 4:1 (v/v) 40% (v/v). The solvent front, which contained glycolipids, was collected and further separated on an adsorption column using a stepped gradient elution with the following solvents: A, acetonitrile; B, acetonitrile/methanol 6:4 (v/v). A hold for 10 min in solvent A was followed by a gradient from solvent A to solvent B in 20 min and finally a hold for 10 min in solvent B. The purified fractions of phospho- and glycolipids from the above separations were separately collected and tested for biological activity. PAF and the examined samples were dissolved in 2.5 mg of bovine serum albumin (BSA)/mL of saline. Thrombin was dissolved in saline. Various concentrations of the examined sample were added into the aggregometer cuvette, and the aggregation induced by the sample was studied in a Chronolog aggregometer (Demopoulos et al., 1979). Experiments with specific inhibitors, 0.7 mM creatine phosphate (CP)/creatine phosphate kinase (CPK), 13 units/mL of saline, 10 μM indomethacin (10% ethanol in water), and 0.1 mM BN 52021 (0.3% DMSO in water), were also performed. These inhibitors were added to washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette. This experiment was carried out according to the method of Lazanas et al. (1988). In desensitization and cross-desensitization experiments platelets were desensitized by the addition of the test lipid to the platelet suspension at a concentration that caused reversible aggregation. A second stimulation was performed immediately after complete disaggregation. The platelet aggregation induced by PAF (1×10^{-10} M, final concentration) was measured as PAF-induced aggregation, in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the examined sample (Demopoulos et al., 1979). Consequently, the plot of percent inhibition (ranging from 0 to 100%) versus different concentrations of the sample is linear. From this curve, we calculate the concentration of the sample that inhibited 50% PAF-induced aggregation. This value is defined as IC_{50}, namely, inhibitory concentration fifty. The IC_{50} values are expressed as milliliters of red wine required for 50% inhibition against PAF. This experiment was also performed with thrombin (0.125 unit/cuvette) to assess the inhibition of thrombin-induced aggregation. Phosphorus determination was carried out according to the method of Bartlett (1959). Hexose determination was carried out according to the method of Galanos and Kapoulas (1965).

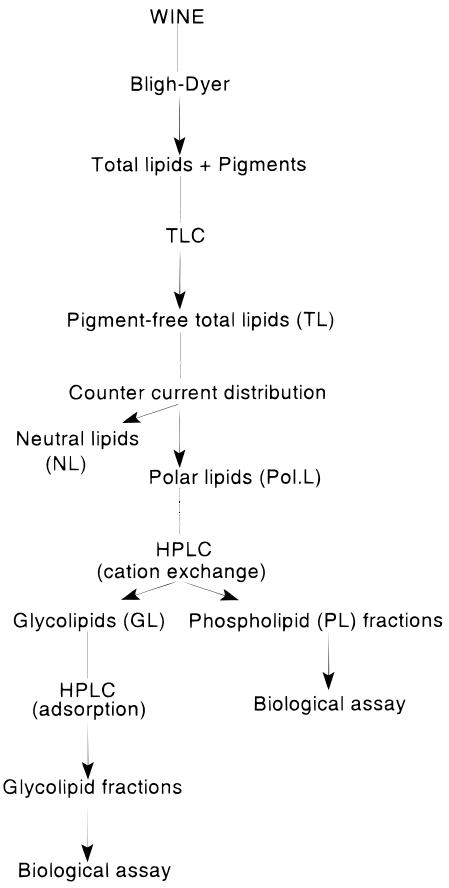

Figure 1. Schematic diagram of the extraction and purification procedure.

RESULTS AND DISCUSSION

The extraction scheme along with the purification procedures are summarized in Figure 1.

The fraction of total lipids free from pigments was separated in neutral and polar lipids by countercurrent distribution (Galanos and Kapoulas, 1962). By this procedure the polar lipid fraction contains glyco- and phospholipids.

Polar lipids were further fractionated on HPLC according to the method of Andrikopoulos et al. (1986).

A typical profile of the polar lipid separation on a cation exchange HPLC column, along with the elution times of standard phospholipids, is shown in Figure 2. Eight phospholipid fractions were collected and tested for their biological activity. The solvent front, namely, the fraction from 0 to ~8 min, which includes mainly glycolipids along with the phosphatidylethanolamine fraction, was collected and further purified on an absorption HPLC column. Even though the purification procedure with the above cation exchange column does not separate glycolipids from a few phospholipids such as phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, it was chosen because it is the most appropriate elution system for the separation of PAF and PAF analogues, on which our work is focused.

A typical profile of the glycolipid fraction separation on an absorption HPLC column, along with the elution times of standard glyco- and phospholipids, is shown in Figure 3. Thirteen lipid fractions were collected and tested for their biological activity. The solvent front, namely, the fraction from 0 to ~10 min, which includes neutral lipids, was collected, pooled with the one

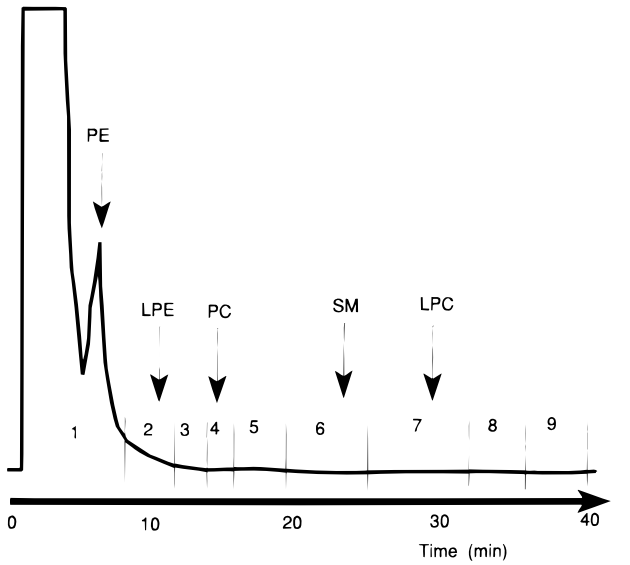

Figure 2. HPLC separation of polar lipids from red wine on a cation exchange column with acetonitrile 60% and methanol/water 4:1 (v/v) 40% (v/v). PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyeline; LPC, lysophosphatidylcholine.

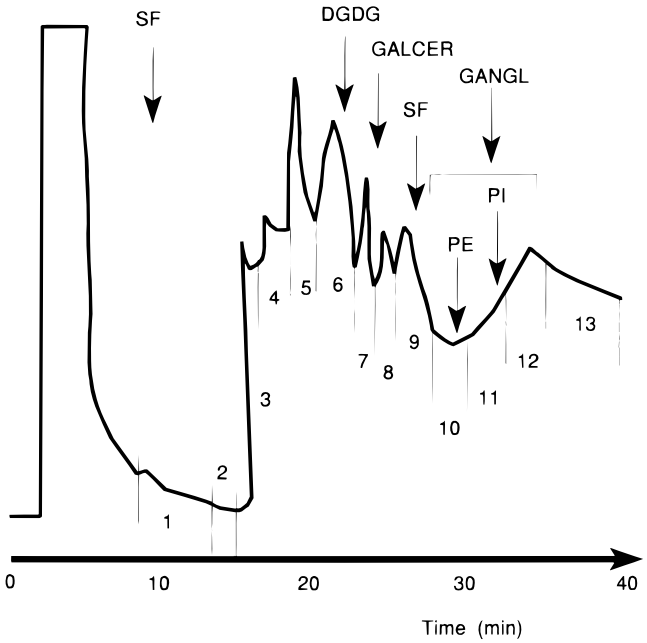

Figure 3. HPLC separation of glycolipids from red wine on an adsorption column with a stepped gradient elution system described in the text. SF, sulfatides; DGDG, digalactosyldiglycerides; GALCER, galactocerebrosides; GANGL, gangliosides; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

obtained from countercurrent distribution, and kept for further study.

All of the HPLC fractions of polar lipids (from both HPLC separations) were tested for their ability to induce washed rabbit platelet aggregation and/or to inhibit PAF-induced and/or thrombin-induced washed rabbit platelet aggregation. The results on the biological activities of each fraction are summarized in Table 1.

All eight fractions collected from the cation exchange column exerted biological activity. Two of them (fractions 2 and 4 of Figure 2) inhibited in a dose-dependent manner PAF-induced aggregation with IC_{50} values of 150 and 233 mL, respectively. Fraction 4 also inhibited

Table 1. Biological Activities of Each Lipid Fraction

fraction	biological action	IC_{50}[a] (mL[b]) against PAF[c]/ thrombin[d]	notes
PL2	inhibition	150	inhibits only PAF
PL3	aggregation		acts through PAF and ADP
PL4	inhibition	233/328	inhibits PAF and thrombin
PL5	aggregation		acts through PAF
PL6	aggregation		acts through PAF and ADP
PL7	aggregation		acts through PAF and ADP
PL8	aggregation		acts through PAF
PL9	aggregation		acts through PAF
GL1	aggregation		acts through PAF and ADP
GL2	inhibition	96/177	inhibits PAF and thrombin
GL3	inhibition	169/99.7	inhibits PAF and thrombin
GL4	inhibition	85/80.2	inhibits PAF and thrombin
GL5	inhibition	47.5/35.6	inhibits PAF and thrombin
GL6	inhibition	58.7/11.6	inhibits PAF and thrombin
GL7	inhibition	86.6	inhibits only PAF
GL8	inhibition	60.4/58	inhibits PAF and thrombin
GL9	inhibition	52/59	inhibits PAF and thrombin
GL10	inhibition	50.1/55.6	inhibits PAF and thrombin
GL11	aggregation		acts through PAF and ADP
GL12	inhibition	17.6/18.2	inhibits PAF and thrombin
GL13	inhibition	123/135	inhibits PAF and thrombin

[a] Inhibitory concentration fifty. [b] IC_{50} expressed as milliliters of red wine required for 50% inhibition against PAF and thrombin. [c] Platelet aggregation induced by PAF 1×10^{-10} M, final concentration. [d] Platelet aggregation induced by thrombin, 0.125 unit/cuvette.

thrombin-induced aggregation with an IC_{50} value of 328 mL. Six of eight fractions (fractions 3 and 5−9 of Figure 2) induced rabbit platelet aggregation with a dose-dependent aggregation pattern similar to that of PAF with the exception of fraction 5, in which a decrease of aggregation was observed at higher concentrations. It seems that fractions 8 and 9 act through PAF receptors for the following reasons: (1) Platelets desensitized to PAF were not aggregated by these lipids and vice versa. (2) Platelets desensitized to these lipids were aggregated by thrombin. (3) The enzymatic system CP/CPK and indomethacin, which specifically inhibit ADP and arachidonic acid-induced platelet aggregation, respectively, have no inhibitory effect on the aggregation induced by these fractions. (4) On the contrary, PAF receptor specific inhibitor BN 52021 does inhibit the aggregation induced by fractions 8 and 9. Fractions 3, 6, and 7 seem to act not only through PAF receptors but also through ADP way since they share the above characteristics with the exception of the inhibition observed by CP/CPK.

Finally, fraction 5 seems to act only through PAF receptors on the basis of data from the above experiments, even though it induced rabbit platelet aggregation with a bell-shaped aggregation pattern. This phospholipid comigrated with synthetic PC on HPLC. Its mass spectrum ions support the structure of the glycophospholipid shown in Figure 4. The biological activity of such a molecule was not unexpected because we have already reported a synthetic glycophospholipid with a similar biological activity (Avramopoulou et al., 1997).

All thirteen fractions collected from the adsorption column exerted biological activity. Two of them (fractions 1 and 11 of Figure 3) induced rabbit platelet aggregation with a dose-dependent aggregation pattern similar to that of PAF. It seems that fractions 1 and 11 act through PAF and ADP receptors: (1) Platelets desensitized to PAF were not aggregated by these lipids and vice versa. (2) Platelets desensitized to these lipids were aggregated by thrombin. (3) Indomethacin had no

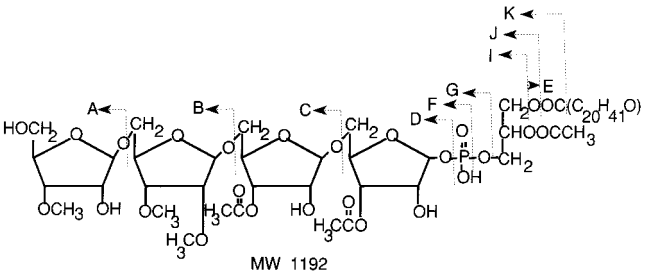

Figure 4. Structure of fraction 5 of glycolipids based on ESMS analysis along with the identified fragments. $[A + H]^+ = 164$; $[A + Na]^+ = 186$; $[A]^- = 163$; $[B + H]^+ = 324$; $[C + H]^+ = 498$; $[498 - OCCH + H]^+ = 456$; $[C + Na + H]^+ = 521$; $[D + Na + H]^+ = 695$; $[E - H]^- = 340$; $[EH + H]^+ = 343$; $[EH + Na]^+ = 365$; $[F + Na + H]^+ = 759$; $[G + H]^+ = 753$; $[I + H]^+ = 852$; $[J + H]^+ = 868$; $[K + H]^+ = 896$; $[MH - OCCH]^+ = 1151$; $[M + Na]^+ = 1215$; $[I - 2H]^- = 849$.

inhibitory effect on the aggregation induced by these fractions. (4) The enzymatic system CP/CPK inhibited their aggregation. (5) PAF receptor specific inhibitor BN 52021 does inhibit the aggregation induced by fractions 1 and 11. Fraction 7 inhibited only PAF-induced aggregation, in a dose-dependent manner, with IC_{50} values of 86.6 mL. Ten of 13 fractions (2−6, 8−10, 12, and 13 of Figure 3) inhibited PAF as well as thrombin-induced aggregation, in a dose-dependent manner. The IC_{50} values for PAF are 96, 169, 85, 47.5, 58.7, 60.4, 52, 50.1, 17.6, and 123 mL, respectively. The IC_{50} values for thrombin are 177, 99.7, 80.2, 35.6, 11.6, 58, 59, 55.6, 18.2, and 135 mL, respectively. These glycolipid fractions exerted almost equal inhibitory activity toward PAF and thrombin.

In fraction 12, which was the most potent inhibitor, phosphorus and hexose determinations were performed. Only sugar was determined, and its IC_{50} value based on glucose determination was of the order of 10^{-4} M, final concentration.

One of the most studied constituents of red wine, resveratrol, is reported to have significant biological action along with its inhibitory activity against thrombin and ADP. Our results demonstrated that standard resveretrol also shows a dose-dependent inhibition of PAF-induced platelet aggregation with an IC_{50} value of 10^{-4} M, final concentration.

In this work, we have demonstrated the existence of a variety of PAF inhibitors along with compounds that antagonize PAF action via its receptors, minimizing the biological effects of PAF in different cells and tissues. Some of the above biologically active compounds of red wine also inhibited thrombin-induced aggregation. These compounds belong to the classes of phospholipids and glycolipids, based on their isolation procedure, their chromatographic behavior, and the results from chemical determinations and structural study.

Our findings shed further light on the nature of the beneficial effects of moderate wine consumption because red wine contains a significant number of lipids with antithrombotic as well as antiatherogenetic action in vitro. These data in correlation with our proposed mechanism of atheromatosis generation may offer a new insight into the well-known "French paradox" from a biochemical point of view.

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