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Competitive kinetics in free radical reactions of cinnamic acid derivatives. Influence of phenoxyl radicals reactions

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Abstract
Relative rates of consumption of caffeic, ferulic and sinapic acids by 2,2′-azobis(2-amidine propane) derived peroxyl radicals has been measured in parallel experiments employing a single substrate and in competitive experiments. Rates of consumption measured in independent experiments at low substrate concentrations (first order limit) follow the order: sinapic > ferulic > caffeic. In agreement with this, in competitive experiments employing simultaneously sinapic and caffeic acid the former compound is consumed considerably faster. On the other hand, in competitive experiments employing ferulic and caffeic acids over a wide range of experimental conditions, caffeic acid is consumed considerably faster than ferulic acid, a result that contrasts with that obtained when both compounds are reacted independently. These apparently anomalous results are interpreted in terms of secondary reactions of the phenol-derived radicals. In particular, hydrogen transfer among phenoxyl radicals and the phenols and fast reactions (disproportionation) of caffeic acid derived radicals could explain these discrepancies.

Keywords: *Peroxyl radicals, cinnamic acids, phenoxyl radicals, competitive kinetics*

Introduction

Evaluation of the reactivity of antioxidants towards reactive oxygen species (ROS) and specifically towards peroxyl radicals is a matter of current interest [1]. In particular, for a given antioxidant XH, the important parameter is the specific rate constant (k_{XH}) of process (1)

$$ROO^{\cdot} + XH \rightarrow ROOH + X^{\cdot} \qquad (1)$$

which could involve a simple hydrogen transfer (as depicted in equation (1)) and/or an electron transfer from XH or its conjugated base. In order to estimate the rate of process (1) from steady state experiments a controlled source of peroxyl radicals and the evaluation of a property related to the efficiency of the process are required. As a free radical source is generally employed, at least in aqueous solutions, the pyrolysis of AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride) [2,3]. The efficiency of the process can be deduced from the rate of XH consumption or from the evaluation of a property directly related to the steady state concentration of peroxyl radicals. Since the evaluation of absolute rate constants is generally cumbersome, most methodologies aimed to this goal only evaluate relative reaction rates, employing a typical antioxidant as reference. If a family of compounds is going to be evaluated, three different experimental approaches can be envisaged to estimate the relative reactivity of two compounds, namely XH and YH:

i. To compare consumption rates obtained in parallel experiments under conditions that warrant the same peroxyl radical steady state concentration;

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ii. To compare consumption rates measured in competitive experiments in which both scavengers are included; and
iii. To evaluate the effect of the additives upon a property directly proportional to the peroxyl radical steady state concentration.

The first two approaches have the disadvantage that they require one to measure the rate of XH and YH consumption, generally by high performance liquid chromatography (HPLC). The third one is the most employed, using luminol chemiluminescence [4,5], the rate of oxygen consumption [6–8] or the bleaching rate of a target molecule as a measure of the peroxyl radical steady state concentration. In this experimental approach, several targets, whose consumption can be easily estimated from absorption or fluorescence spectroscopies [9–17], have been employed as reporters of the steady state radical concentration.

In spite of the large number of works that employ these techniques in the evaluation of antioxidant capacities in families of related compounds, no comparison of results obtained by the three procedures has been reported. In the present work we have selected three closely related compounds recognized as relevant antioxidants, ferulic, caffeic and sinapic acids, and study their consumption in parallel and competitive experiments by HPLC. These data, together with that reported regarding their capacity to reduce the rate of a target molecule consumption, allow a comparison of k_{XH}/k_{YH} values obtained from different experimental approaches. Differences obtained are explained in terms of the relevance of secondary reactions involving phenoxyl radicals.

Experimental

Chemicals

AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride), ferulic acid (4-hydroxy-3methoxycinnamic acid), sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid), and caffeic acid (3,4-dihydroxycinnamic acid) were purchased from Sigma-Aldrich (St. Louis, MO) and employed as received.

HPLC analysis

Cinnamic acid derivatives consumptions were evaluated by HPLC. Chromatograms were obtained using an Agilent 1100 Series HPLC (Palo Alto, CA), equipped with a μbondapack C18, 10 μm, 3.9 × 300 mm HPLC column (Waters) and a diode array detector (DAD G1315A). For the quantification of cinnamic acids derivatives wavelength of 312 nm was used. Phosphate (KH_2PO_4, 10 mM adjusted to pH 2.6 with HCl)/acetonitrile (80/20 V/V), was employed as mobile phase (isocratic elution). The flow rate was 0.8 mL/min. All experiments were carried out in duplicate or triplicate.

Oxidative degradation of cinnamic acids

To evaluate the interaction between cinnamic acids and peroxyl radicals, sinapic, ferulic and/or caffeic acid were incubated at 37°C in the presence of 10 mm of AAPH in phosphate buffer (10 mM), pH 7.0, under aerobic conditions. At different times, aliquots were taken and immediately injected in the HPLC. No concentration changes were observed in control experiments carried out in the absence of AAPH. Results were analysed with the HPLC-computer-coupled ChemStation LC 3D program.

Experiments employing a single free radical scavenger were carried out with substrate concentrations in the 5–300 μM range. Competitive experiments were performed at concentrations of 10 and 100 μM of each acid. Initial consumption rates were obtained from the slope, at $t = 0$, of the best fitting curve of concentration vs time plots.

Results

Consumption rates measured employing a single substrate (parallel experiments)

Incubation of a single cinnamic acid derivative in the presence of AAPH leads to its progressive consumption. Results obtained employing 10 μM sinapic, caffeic or ferulic acids in separate experiments are shown in Figure 1. These data show that, under these conditions, sinapic and ferulic acids are considerably more reactive than caffeic acid. This is stressed when initial consumption rates, obtained at different concentrations, are plotted against the initial substrate concentration (Figure 2). These plots show the

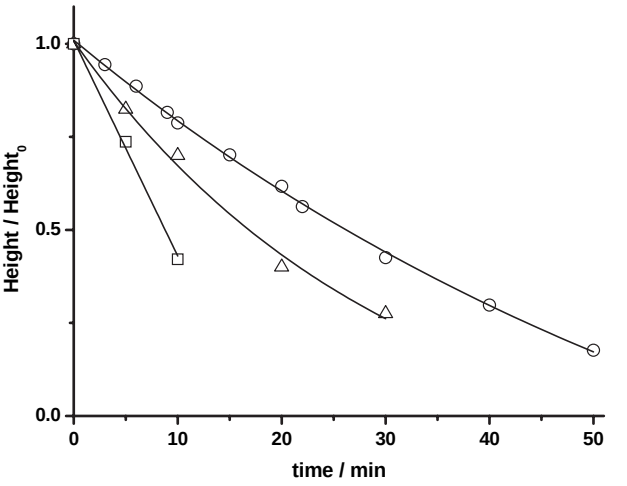


Figure 1. Time-course of the relative height of HPLC peaks of ferulic (△), caffeic (○) and sinapic (□) acids, measured in parallel experiments. The cinnamic acid derivatives (10 μM) were incubated at 37°C in the presence of AAPH (10 mM) in air-saturated solutions.

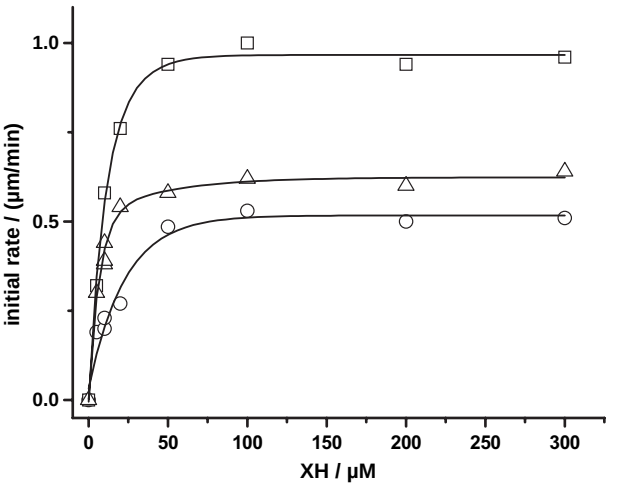


Figure 2. Initial rate of the cinnamic acid derivatives consumption elicited by AAPH (10 mM) as a function of the tested compound concentration. Ferulic (△), caffeic (○) and sinapic acid (□) were incubated at 37°C in the presence of AAPH (10 mM) in air-saturated solutions.

expected profiles, with near first order kinetics at low concentrations and zero order kinetics at high substrate concentrations. In the latter conditions (zero order kinetic limit), the fate of all peroxyl radicals is determined by the substrate and/or their derived radicals [6]. The differences in rates observed in the high concentration range are then related to the stoichiometry of the scavenging process and are unrelated to the reactivity of the tested compound towards peroxyl radicals.

From parallel experiments (Figures 1 and 2) k_{XH}/k_{YH} values can be estimated from the initial rates of XH and YH consumption. In fact, under conditions of first order kinetics, e.g. at low substrate concentrations, the absolute rate of consumption of XH and YH elicited by peroxyl radicals will be given by:

$$d[XH]/dt = a\ k_{XH}[XH] \quad (2)$$

and

$$d[YH]/dt = a\ k_{YH}\ [YH] \quad (3)$$

In these equations, the factor a represents the steady state concentration of peroxyl radicals and is determined by the AAPH concentration and temperature, independently of the added scavenger and its concentration. Comparison of the rates obtained under similar conditions allows one then to obtain the relative reactivities through equation (4).

$$k_{XH}/k_{YH} = (d[XH]/dt)/\ (d[YH]/dt) \quad (4)$$

Relative reaction rates estimated by this procedure are given in Table I. The data of this table would indicate that, in parallel experiments at low concentrations, ferulic acid is almost twice as reactive towards peroxyl radicals than caffeic acid. However, this conclusion is based on equation (4) that disregards secondary reactions of the phenol derived radicals that could affect the rate of the parent compound consumption. In particular, with caffeic acid, a process such as

$$2HO{-}Ph{-}O^{\cdot} \rightarrow HO{-}Ph{-}OH + O{=}Ph{=}O \quad (5)$$

could reduce by a factor of two the parent phenol consumption. Formation of the orto-quinone in the caffeic/AAPH system is supported by a time-dependent increase in the absorbance of the sample at 400 and 240 nm [18,19]. If reaction (5) predominates,

$$k_{caffeic}/k_{ferulic} = 2(d[caffeic]/dt)/(d[ferulic]/dt) \quad (6)$$

and the experimental data allow one to conclude that

$$0.9 \leq k_{ferulic}/k_{caffeic} \leq 1.8 \quad (7)$$

The data given in Figures 1 and 2 show that sinapic acid is consumed faster that ferulic and caffeic acids. Values of $k_{sinapic}/k_{caffeic}$ are collected in Table I. From reaction rates measurements in parallel experiments it must then be concluded that, regarding their reactivities towards peroxyl radicals,

$$k_{sinapic} > k_{ferulic} \geq k_{caffeic}$$

Consumption rates measured in competitive experiments employing two substrates

In this experimental approach, XH and YH compete for the peroxyl radicals according to:

$$ROO^{\cdot} + XH \rightarrow XH\text{-Consumption} \quad (8)$$

$$ROO^{\cdot} + XH \rightarrow YH\text{-Consumption} \quad (9)$$

Table I. $k_{ferulic}/k_{caffeic}$ and $k_{sinapic}/k_{caffeic}$ values estimated from different experimental approaches.

Methodology	Ferulic/caffeic	Sinapic/caffeic	References and comments
Ratio of initial slopes in parallel experiments carried out at low concentration (10 μM)	1.8	2.9	Present work, equation (4)
Ratio of initial slopes in competitive experiments. Low concentration (10 μM)	0.04	9.0	Present work, equation (11)
Ratio of initial slopes in competitive experiments. High concentration (100 μM)	0.3*	4.9	Present work, equation (11)
Protection experiments employing crocin as target	0.23	1.5	[15,16]
Protection experiments employing c-Phycocyanin (cPC) as target	0.5	2	[11]. Secondary damage of c-PC by antioxidant-derived radicals
Protection experiments employing pyranine as target	0.072	—	[19,21]. Value associated to a repair mechanism

* This value represents an upper limit bound to a large uncertainty due to the very slow consumption of ferulic acid (Figure 4B).

Under steady state conditions, the relative rates of consumption of the additives will be given by

$$d[XH]/d[YH] = (k_{XH}/k_{YH})([XH]/[YH]) \quad (10)$$

allowing a direct evaluation of k_{XH}/k_{YH}

$$k_{XH}/k_{YH} = (d[XH]/d[YH])([YH]/[XH]) \quad (11)$$

Eqnuations (10) and (11) assume that relative rates of XH and YH consumption are not influenced by the fate of the radicals ($X^{\bullet}$ and $Y^{\bullet}$) generated in processes represented by equations (8) and (9), respectively.

Competitive experiments carried out employing caffeic and sinapic acids as substrates show a considerably faster consumption of sinapic acid. Typical results are shown in Figure 3 . From equation (11) it can then be concluded that $k_{sinapic}/k_{caffeic} > 1$ (Table I). In this regard, the conclusion reached from competitive experiments agrees, at least qualitatively, with that reached from a comparison of the rates obtained in parallel experiments.

Consumptions of caffeic and ferulic acids elicited by their joint incubation in presence of AAPH are shown in Figure 4 at low (10 μM) and high (μm) concentration of each substrate, respectively. In both conditions, caffeic is consumed faster than ferulic acid. If equation (11) is applied to these data, it must be concluded that

$$k_{caffeic}/k_{ferulic} >> 1,$$

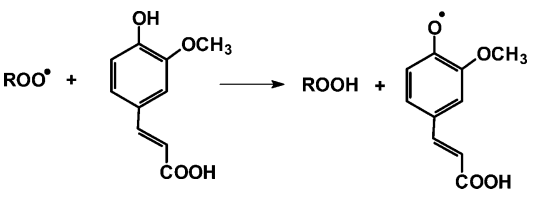
a result in disagreement with that obtained when rates measured in parallel experiments are considered.

Discussion

Table I shows $k_{ferulic}/k_{caffeic}$ and $k_{sinapic}/k_{caffeic}$ values obtained in the present work in parallel and competitive experiments under different experimental conditions. In this table are included values derived from protection experiments using c-phycocyanin, crocin or pyranine as target molecule.

The most remarkable aspect of these data is the large differences in $k_{ferulic}/k_{caffeic}$ ratio obtained employing different experimental procedures. In particular, the apparent high reactivity of ferulic acid evaluated in parallel experiments is noticeable.

The initial interaction of peroxyl radicals with caffeic and ferulic acid leads to the oxygen centred radicals:



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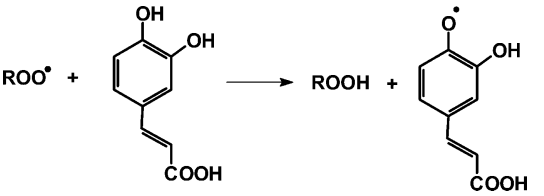
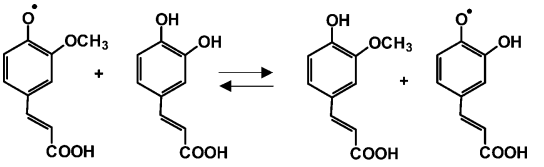


(13)

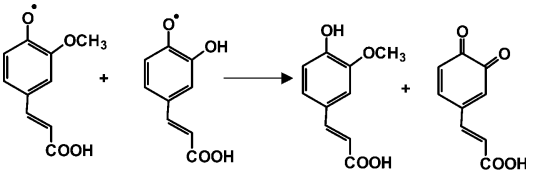
In experiments employing caffeic acid as the only substrate, an efficient disproportionation of *a*-hydroxyphenoxyl radicals produced in the process represented by equation (13) could reduce its consumption rate. This could introduce up to a factor two of error in the application of equation (4). Even taking this into account, the data obtained in parallel experiments would indicate that ferulic acid is at most as reactive as caffeic acid, a conclusion that contrasts with that derived from competitive experiments.

In competitive experiments, faster radical–radical reactions of the radical derived from caffeic acid can influence the additives consumption rates. In particular, hydrogen exchange processes between phenols and their derived radicals [20,21]



(14)

and disproportionation reactions leading to stable products, such as



(15)

would preserve the monophenol, decreasing its rate of consumption and increasing the consumption of the compound (caffeic) that can lead to the corresponding quinone. This could be particularly important since it can be expected and equilibrium constant $K_{14} \geq 1$ [22,23]. Furthermore, reduced rate of radical–radical reactions, due to steric hindrance of the vicinal methoxy groups, would further preserve the sinapic acid [24]. Under these conditions, relative rates of consumption would be almost unrelated to reactivities towards peroxyl radicals, being mainly determined by the equilibrium among phenoxyl radicals and the rate constants of their secondary reactions. In particular, enhanced rates of consumption would be obtained for those compounds whose radicals can readily react to give stable products, such as the quinone derived from caffeic acid. This precludes application of equation (11) under these conditions.

The contribution of these processes would depend on the experimental conditions and could explain the variety of results present in Table I and the apparent

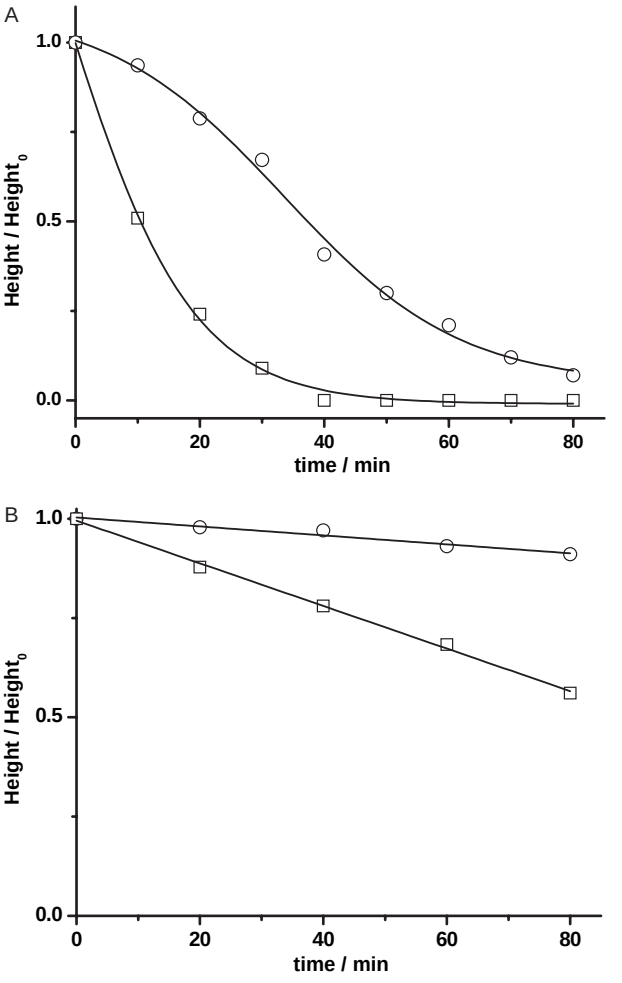


Figure 3. Competitive experiments at low (A) and high (B) additive concentrations. Relative heights of HPLC peaks of caffeic (○) and sinapic (□) acids vs incubation time (min). The cinnamic acid derivatives at 10 μM (A) and 100 μM (B) were incubated at 37°C in the presence of AAPH (10 mM) in air-saturated solutions.

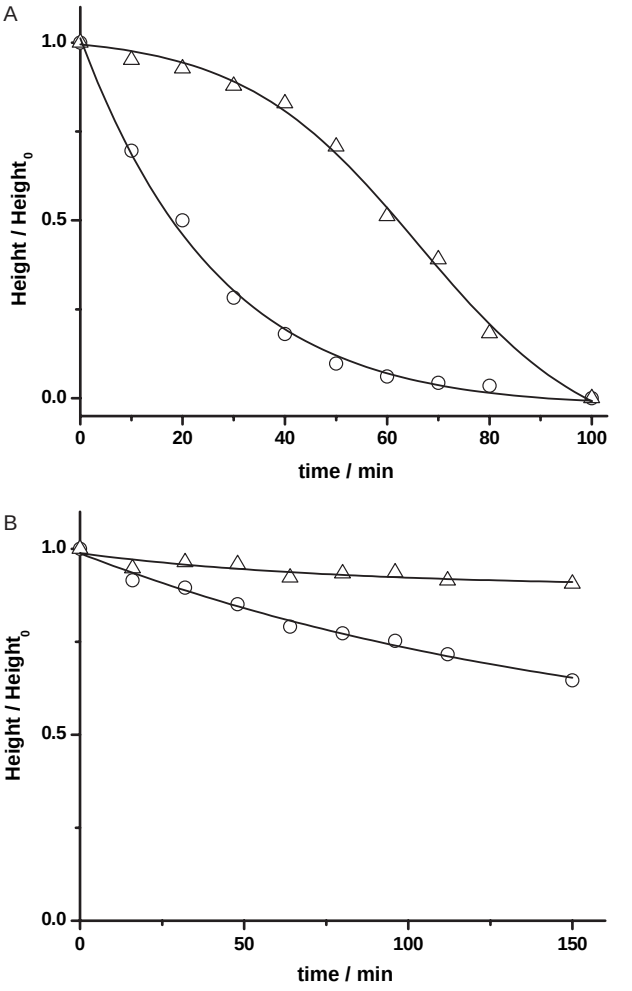


Figure 4. Competitive experiments at low (A) and high (B) additive concentrations. Relative heights of ferulic (△) and caffeic (○) acids HPLC peaks are plotted against the incubation time. The cinnamic acid derivatives at 10 μM (A) and 100 μM (B) were incubated at 37°C in the presence of AAPH (10 mM) in air-saturated solutions.

controversy of published data on the relative reactivity of ferulic and caffeic acids towards peroxyl radicals [7,8].

On the other hand, in competitive experiments sinapic acid remains more reactive than caffeic acid, implying a process such as that depicted in equation (16)

$$\text{sinapic}^{\cdot} + \text{caffeic}^{\cdot} \rightarrow \text{sinapic} + \text{O=Ph=O} \qquad (16)$$

is less important than the reaction represented in equation (14). Two reasons can be proposed for this lack of significance. In the first place, an equilibrium such as

$$\text{caffeic}^{\cdot} + \text{sinapic} \rightleftarrows \text{caffeic} + \text{sinapic}^{\cdot} \qquad (17)$$

would be displaced towards the right, due to the weakness of the phenolic hydrogen bond in sinapic acid [23]. This would reduce the concentration of caffeic acid derived radicals, reducing the importance of reaction (16). In the second place, it can be expected that process (16) be less exothermic than (15), a factor that could further reduce the rate of the process. In fact, largest values of $k_{\text{sinapic}}/k_{\text{caffeic}}$ are derived from competitive experiments (Table I). This could be attributed to the displacement towards the right of the equilibrium depicted by equation (17). In this regard, it is interesting to consider that the difference in bond dissociation energy (BDE) between sinapic and caffeic acids (~4 kcal/mole in protic solvents) [23] must influence more the enthalpy of equilibrium (17) than the activation energies of the reactions of the cinnamic derivatives with peroxyl radicals.

Reactivity ratios obtained in the present work poorly correlate with those obtained by other methodologies based on the protection of target molecules (Table I). A quantitative interpretation of the differences reported in this table is difficult due to the multiplicity of secondary reactions that could affect the estimation of relative rate constants. For example, it has been proposed that pyranine protection afforded

by a given phenolic compound is unrelated to the substrate reactivity, being determined by a repair mechanism [22,25]. Similarly, an enhanced caffeic acid consumption could be due to a reaction such as [26]:

$$\text{caffeic}^{\bullet} + O_2 \rightarrow O_2^{\bullet -} + \text{quinone} \qquad (18)$$

This reaction, besides selectively removing caffeic acid, would lead to secondary phenols and/or target molecules consumption promoted by the hydroperoxyl radical (or superoxide) reactions. The relevance of these reactions, as well as that of those previously described, would depend of the experimental setting (methodology and substrate concentrations) leading to complex relationships between substrate reactivity towards peroxyl radicals and the rates of their consumption.

Conclusions

Competitive experiments in which is measured the consumption of two substrates appear as the simplest way of estimating the relative reactivity of free radical targets. Nevertheless, special care must be taken when target derived radicals present widely different radical–radical reaction rates, regenerate the parent compound and/or interchange between them.

Acknowledgements

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