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Tetrahedron Letters
Tetrahedron Letters 47 (2006) 6611–6614

Hydroxycinnamic acid clustered by a calixarene platform: radical scavenging and antioxidant activity

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Received 26 May 2006; revised 3 July 2006; accepted 6 July 2006
Available online 28 July 2006

Abstract—Novel hydroxycinnamic acid–calix[4]arene hybrids **4** and **5** were synthesized. Their radical scavenging and antioxidant activities were determined by using DPPH· radical and AIBN·–induced linoleic acid peroxidation test, respectively. Preliminary studies showed that compounds **4** and **5** possess enhanced activity with respect to the corresponding hydroxycinnamic acid and phenetidine derivative. Kinetic solvent effects were taken in account to understand the different antioxidative behaviour of the synthesized compounds.

Hydroxycinnamic acids (HAs) and their derivatives have various properties which may be of importance for the remediation of many diseases. Their activities of biomedical interest (antibacterial,[1a] antiviral,[1b] antiinflammatory,[1c] immunostimulatory,[1c] antiatherogenic,[1d] antiproliferative,[1e] neuroprotective,[1e] and radiosensitizing[1f]) are related to their capability to act as antioxidants, metal chelants, and enzyme inhibitors or to link specific receptors.[2] In particular, caffeic (CA) and sinapic (SA) acids are natural antioxidants widely spread in the plant kingdom, in various beverages and foodstuffs, to which they impart an atherosclerosis preventive function.[3]

Antioxidant activity is a multifactorial event. As concerns the HA derivatives, the main factors which modulate this property are propensity to radicals formation, electron-donating or -withdrawing substituents on catechol moieties, involvement of other H-donating groups (–NH, –SH), chemical stability, and lipophilicity.[4]

The interest toward these compounds prompted us to synthesize novel derivatives by using a rigid molecular platform for the presentation of a HA cluster. This, in principle, could generate novel molecular architectures with amplified recognition, radical scavenging and antioxidant activities in comparison to a single HA unit.

We decided to use a calix[4]arene scaffold, macrocyclic molecule with unique tridimensional structures (*cone, partial cone, 1,2-* and *1,3-alternate* conformation).[5] Due to their synthetic versatility, low cost and limited-toxicity, calixarenes have been widely used in supramolecular chemistry as building blocks or molecular scaffolds for the construction of various receptors.[6]

Recently, a cluster effect in molecular recognition phenomena has been demonstrated for multivalent calix[4,8]arene derivatives.[7]

Here we wish to report free-radical scavenging and antioxidative activities of calix[4]arene derivatives exposing four units of hydroxycinnamic acid in *all-syn* orientation.

The synthesis[8] of caffeoyl- and sinapyl-calix[4]arene derivatives **4** and **5** is depicted in Scheme 1. Addition of the tetra-aminocalix[4]arene derivative **1**[9] to a DMF solution of the corresponding acetylated hydroxycinnamic acid (**2** or **3**), in the presence of PyBOP and DIPEA, afforded the acetylated intermediate compounds in 50% isolated yield.

Keywords: Calixarene; Hydroxycinnamic acids; Radical scavenging and antioxidant activity.
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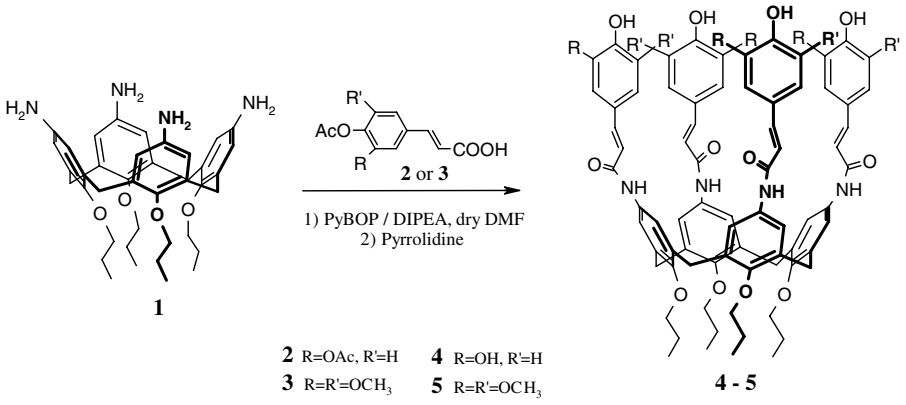
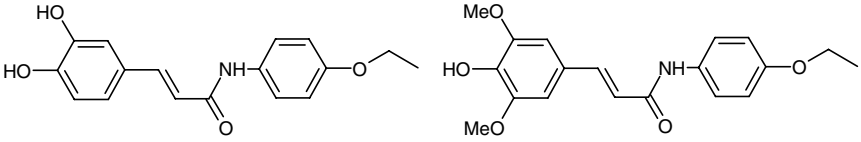
doi:10.1016/j.tetlet.2006.07.021



Scheme 1.

De-acetylation, in the presence of pyrrolidine, gave pure hybrid compounds **4** and **5** in quantitative yields. Their structures were characterized[10] by NMR and ESI-MS spectra.

In order to evaluate the potentially enhanced radical scavenging and antioxidant activities of the multivalent compounds (**4** and **5**), we prepared the *p*-phenetidine derivatives **6** and **7**,[11] as reference compounds. In fact, they can be assimilated to 1/4 of the corresponding hybrid structures **4** and **5**.



6 **7**

Free radical scavenging activity was determined by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$).[12]

The rate constant values (methanol as solvent, $k_1 > 1.5 \times 10^4$) and the stoichiometric factors n (number of DPPH$^{\cdot}$ radicals quenched per antioxidant molecule) for **4** ($n = 7.7$) and **5** ($n = 2.7$) were indicative of their high radical quenching capability. Interestingly, compounds **6** and **7**, the free caffeic acid, and the free sinapic acid showed lower values of n (1.94, 1.2, 2.2, 1.0, respectively) than the hydroxycinnamic acid–calixarene conjugates.

The very high rate constant values suggest that reactions of compounds **4** and **5** with the DPPH$^{\cdot}$ radical, in a strong hydrogen bond accepting solvent like methanol, occur by a fast electron transfer (ET) mechanism. Consequently, the hydrogen atom abstraction (HAT) process is a marginal reaction, occurring very slowly. This agrees with the results reported by Foti et al.[13] for free caffeic acid, sinapic acid, and their methyl ester derivatives, and by Ingold and Litwinienko[14] for hindered and non hindered phenols.

The antioxidant activity of compounds **4**–**7** was determined by the in vitro model using azo-bis-isobutyryl-nitrile (AIBN)–induced linoleic acid peroxidation.[15] The alkylperoxyl-radicals (HLOO$^{\cdot}$) generated from AIBN are similar to the radicals formed in biological systems. These radicals are responsible for the peroxidation of human low-density lipoproteins (LDL), which are implicated in diseases such as atherosclerosis and cancer.

Table 1 reports the values of k_{inh}/k_p, indicative of antioxidant efficiency, obtained in acetonitrile for compounds **4**–**7**. Compounds **4** and **5** showed absolute rate constants k_{inh} and stoichiometric factors n to be higher with respect to the reference compounds **6** and **7**, but a fourfold increase was not observed.[16] This could be due to the particular spatial geometry that the calixarene scaffold confers to the HA units.

The k_{inh} value of sinapic acid derivative **5** appears to be particularly high, by fourfold, in comparison with the corresponding caffeic acid derivative **4**. The same ratio was also observed for phenetidine derivatives **7** and **6**.

This behaviour might appear surprising considering that measured O–H bonds dissociation enthalpy (BDE) for free sinapic and caffeic acids are very similar (80–

Table 1. Antioxidant efficiencies k_{inh}/k_p,[a] absolute rate constant[b] k_{inh} and stoichiometric factors n[a] for the reaction of ArOH + HLOO$^{\cdot}$ in acetonitrile at 50 °C

Compound	$k_{inh}/k_p \times 10^3$	$k_{inh} \times 10^4$ ($M^{-1} \times s^{-1}$)	n
4	3.7	18.5	5.9
5	14.5	72.5	2.7
6	2.7	13.6	1.6
7	11.5	57.5	1.1

[a] Experimental error ±10%. Average of the values of three to five experiments.

[b] Calculated with $k_p = 50\ M^{-1} \times s^{-1}$ for linoleic acid.

81 kcal/mol).[17] However, this unexpected behaviour can be understood taking into account the kinetic solvent effects. Measurements in cyclohexane[18] showed that the k_{inh} of compounds **6** (1.1×10^6) and **7** (6×10^5) differ slightly (factor of 1.6). Therefore, the lower k_{inh} values of caffeoyl derivatives **4** and **6**, with respect to sinapyl derivatives **5** and **7**, measured in acetonitrile, are ascribable to the stronger interaction of the caffeoyl moiety with the polar solvent.

In conclusion, the present work describes the first examples of hydroxycinnamic acid–calixarene hybrids with effective radical scavenging and antioxidant activities. These derivatives could be regarded as novel molecular constructs with defined shape and high density antioxidant surface, potentially useful for biological applications.

Acknowledgements

The authors thank Professor M. Piattelli for a critical reading of the manuscript and Dr. M. C. Foti for helpful discussions. Thanks are also due to Mr. A. Renda (ICB, CNR) for ESI-MS spectra.

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8. *Procedure for the preparation of compounds* **4** and **5**: A solution of **2** or **3** (0.675 mmol), PyBOP (0.675 mmol) and DIPEA (120 μL) in dry DMF (1.6 mL) was stirred for 5 min at 0 °C. Tetra-aminocalix[4]arene **1** (0.153 mmol) was added and the solution was stirred for 30 min at 0 °C and for 18 h at room temperature. The reaction mixture was poured into 10 mL of 1 N HCl. The insoluble material was collected by filtration, washed with water, dried and purified by TLC on silica gel (95:5 CH_2Cl_2/MeOH) to give the amide intermediate. Amide derivative (50 mg) was dissolved in pyrrolidine (100 μL) at room temperature. After 1 h, a solution of 1 N HCl (10 mL) was added and the insoluble material was collected by filtration and dried to give de-acetylated compounds **4** or **5**.
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10. Satisfactory microanalytical and spectral data were obtained for all new compounds. NMR spectra were taken at 400 MHz. Compound **4**: ^{1}H NMR (MeOD, 295 K), 1.05 (t, CH_3, $J = 7.3$ Hz, 12H), 1.98 (m, CH_2, 8H), 3.15 and 4.50 (AX system, $ArCH_2Ar$, $J = 13.1$ Hz, 8H), 3.88 (t, OCH_2, $J = 7.0$ Hz, 8H), 6.38 (d, CH=CH, $J = 15.5$ Hz, 4H), 6.62 (d, *caffeoyl*-ArH, $J = 8.0$ Hz, 4H), 6.8 (d, *caffeoyl*-ArH, $J = 8.0$ Hz, 4H), 6.90 (s, *caffeoyl*-ArH, 4H), 7.04 (s, ArH, 8H), 7.40 (d, CH=CH, $J = 15.5$ Hz, 4H), ^{13}C NMR (MeOD, 295 K), 10.8 (q), 24.4 (t), 32.2 (t), 78.1 (t), 115.4 (d), 116.5 (d), 118.9 (d), 121.7 (d), 122.1 (d), 128.3 (s), 134.1 (s), 136.3 (s), 142.9 (d), 146.5 (s), 148.7 (s), 154.5 (s), 167.1 (s, C=O); ESI-MS (*m*/*z*): calcd for $C_{76}H_{75}N_4O_{16}$ $[M\text{-}H]^-$ 1299.52, found 1299.3. Compound **5**: ^{1}H NMR (MeOD, 295 K), 1.03 (t, CH_3, $J = 7.3$ Hz, 12H), 1.96 (m, CH_2, 8H), 3.16 and 4.51 (AX system, $ArCH_2Ar$, $J = 12.8$ Hz, 8H); 3.75 (s, OCH_3, 24H), 3.88 (br t, OCH_2, 8H), 6.48 (d, CH=CH, $J = 15.2$ Hz, 4H), 6.69 (s, *sinapyl*-ArH, 8H), 7.07 (s, ArH, 8H), 7.43 (d, CH=CH, $J = 15.2$ Hz, 4H), ^{13}C NMR (Acetone-d_6, 295 K) 10.7 (q), 23.4 (t), 32.0 (t); 56.5 (q), 77.6 (t), 106.3 (d), 120.6 (d), 126.9 (s), 134.6 (s), 135.7 (s), 138.8 (s), 141.5 (d), 148.8 (s), 153.5 (s), 162.9 (s, C=O); ESI-MS (*m*/*z*): calcd for $C_{89}H_{91}N_4O_{20}$ $[M\text{-}H]^-$ 1475.62, found 1475.9.
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12. Kinetic experiments were performed as reported on Ref. 13; the stock solutions of compound (**4**–**7**) were prepared in MeOH. The stoichiometric factors *n* were calculated

(with [DPPH$^{\cdot}$] > [Antioxidant]) from the following equation: $n = (\Delta A_{515}/\varepsilon_{515})/[\text{Antioxidant}]$ where ΔA_{515} is the absorbance difference between the initial and stationary state of DPPH$^{\cdot}$ solution, $\varepsilon_{515} = 10865\ M^{-1}\,cm^{-1}$, and [Antioxidant] is the concentration of antioxidant in cuvette at time zero. The rate constants k_1 were thereby obtained from the decay traces using the initial rates at 298 K.

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15. Kinetic experiments were performed as reported by Foti, M. C.; Ruberto, G. *J. Agric. Food Chem.* **2001**, *49*, 342, the stock solutions of antioxidant (**4**–**7**) were prepared in EtOH immediately prior to use. The antioxidant efficiencies k_{inh}/k_p and absolute rate constant k_{inh} were determined from the following equation: $k_{inh}/k_p = -(\varepsilon_{234}/1.19)[H_2L]\ (\ln(1-t/\tau))/\Delta A_{234}$ where ε_{234} is the molar extinction coefficient of HLOOH at 234 nm, $[H_2L]$ is the linoleic acid concentration, ΔA_{234} is the increase of absorbance at time t, and τ is the induction period (obtainable from the curve A_{234} vs. time). The value of k_p for linoleic acid is known: $k_p \sim 50\ M^{-1} \times s^{-1}$. The stoichiometric factors n were determined from the following equation: $\tau = (n \cdot [\text{Antioxidant}]_{t=0})/R_i$ where $[\text{Antioxidant}]_{t=0}$ is the concentration of antioxidant in cuvette at time zero, and R_i is the rate of radical production of AIBN: $R_i = 2k_d e[\text{AIBN}]$. The factor 0.97 was used to correct the concentration of all compounds for the thermal expansion of the solvent at 50 °C.
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18. Because the insolubility of compounds **4** and **5** in cyclohexane, the kinetic measurements were only performed for compounds **6** and **7** (the method is reported in Ref. 15).