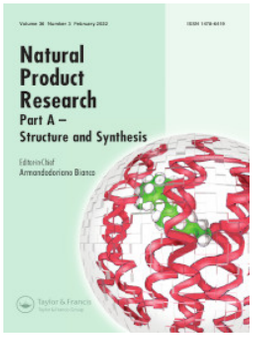

Natural Product Research

Formerly Natural Product Letters

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gnpl20

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To cite this article: Andrea Brizzolari, Mario C. Foti, Luciano Saso, Pierangela Ciuffreda, Jelena Lazarević & Enzo Santaniello (2022): Evaluation of the radical scavenging activity of some representative isoprenoid and aromatic cytokinin ribosides (N^6-substituted adenosines) by *in vitro* chemical assays, Natural Product Research, DOI: 10.1080/14786419.2022.2037590

To link to this article: https://doi.org/10.1080/14786419.2022.2037590

NATURAL PRODUCT RESEARCH
https://doi.org/10.1080/14786419.2022.2037590

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SHORT COMMUNICATION

Evaluation of the radical scavenging activity of some representative isoprenoid and aromatic cytokinin ribosides (N^6-substituted adenosines) by *in vitro* chemical assays

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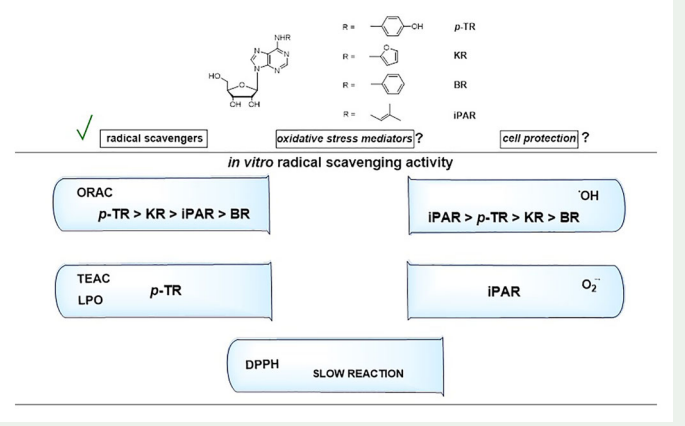
ABSTRACT

Cytokinins are naturally occurring adenine derivatives whose physiological role is that of growth regulators in plants and that show also many other activities either in plants and in mammalian cells. In plants, they can be found mainly as free bases (N^6-substituted adenines, CKs), but also as the corresponding N^9-ribosides (N^6-substituted adenosines, CKRs). In mammalian cells, CKRs are, in general, more active than CKs. In order to evaluate the intrinsic *in vitro* antioxidant capacity of some significant CKRs, their scavenging activity against synthetic radicals that are at the basis of well-established antioxidant assays (ORAC, TEAC, DPPH) has been evaluated. The results of the *in vitro* scavenging activity of biologically relevant radicals such as hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{\cdot-}$) and lipid peroxides (R-$OO^{\cdot}$) are reported and discussed.



ARTICLE HISTORY
Received 11 December 2021
Accepted 25 January 2022

KEYWORDS
Oxidative stress; cytokinins (bases and ribosides); *in vitro* antioxidant activity; radicals; chemical assays

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2022.2037590.

1. Introduction

Cytokinins are adenine derivatives substituted at the N^6-position (structure **1**, Supplementary material, Figure S1) that naturally occur as free bases in plants where they physiologically exert a hormonal activity promoting cell division, growth and retardation of senescence, thus influencing almost each part of plant developmental stages (Mok and Mok 2001). Due to their peculiar effect on cell division and differentiation in plant cells, cytokinins were also studied for their effect on the differentiation of human cells, both normal and malignant (Honma and Ishii 2002). The N^9-ribosides of CKs (CKRs) showed a higher biological activity, so that became also potential candidates for treating a variety of cancers (Voller et al. 2017, 2019).

It has been proposed that the activities of cytokinins can be associated to their capacity to lower the oxidative stress interacting with biological regulators of oxidative stress (Othman et al. 2016; Hönig et al. 2018). An intrinsic antioxidant effect due to their structure cannot be excluded and this aspect has been recently investigated by Brizzolari et al. (2016) for four natural N^6-adenines (CKs, **1a–d**, Supplementary material, Figure S1). We have now evaluated the radical scavenging activity of the corresponding N^9-ribosides (CKRs, **2a–d**, Supplementary material, Figure S1) by *in vitro* assays that quantify the attitude of the compounds to react with synthetic radicals (Supplementary material, Figure S2) according to hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms (Huang et al. 2005), i.e. ORAC (HAT), TEAC (ET) and DPPH [mixed HAT/ET (Foti et al. 2004)] assays.

The *in vitro* the scavenging activity of CKRs **2a–d** (Supplementary material, Figure S1) against biologically relevant radicals such as the hydroxyl radical ($HO^{\bullet}$) was established by the 2-deoxyribose (2-DR) degradation assay (Halliwell and Gutteridge 1981; Aruoma 1994). Finally, all CKs and CKRs were *in vitro* assayed against lipid peroxides (Lazarević et al. 2020) and superoxide anion radical ($O_2^{\bullet-}$, Yen and Duh 1994).

2. Results and discussion

2.1. ORAC, TEAC and DPPH assays of CKRs

The results of ORAC assay for CKRs (Supplementary material, Figure S3) show that, in the range of 1 μM–5 μM, *p*-TR is the most active CKR, most likely for the presence of the phenolic OH group, which readily donates H-atom to AAPH peroxyl radical ($AAPH^{\bullet} = ROO^{\bullet}$) generated in the ORAC assay.

Other H-atom donors that could react with $ROO^{\bullet}$ are present in the structure of CKRs, but the N^6-H group should play a major role (Steenken 1989). In CKRs, this NH group is bound to structurally diverse groups, thanks to which different reactivity with $ROO^{\bullet}$ can be observed (*p*-TR > KR > iPAR > BR).

In the TEAC assay, at a concentration range 0.5 μM to 5 μM, only *p*-TR exhibited a scavenging activity that was nearly identical to that of *p*-T (Brizzolari et al. 2016). The overall results obtained from the examined CKs and CKRs suggest that only the phenol moiety of *p*-T and *p*-TR can react with $ABTS^{\bullet+}$ radicals by an ET mechanism. Interestingly, in the concentration range 0.5 μM to 5 μM, the antioxidant capacity of *p*-T and *p*-TR is higher than that of Trolox (Supplementary material, Figure S4).

This could be related to a limited steric accessibility of the bulky $ABTS^{\bullet+}$ radical to the congested 6-hydroxy group in Trolox, that is flanked by two methyl groups.

Finally, neither CKs nor CKRs showed any scavenging ability against $DPPH^{\bullet}$ in the maximum concentration ranges of reagents and substrate that could be reached in ethanol.

2.2. Scavenger activity of CKRs against chemically generated hydroxyl radicals $HO^{\bullet}$ and superoxide radicals ($O_2^{\bullet-}$)

The reactivities of all four CKRs with the chemically generated hydroxyl radicals ($HO^{\bullet}$) were higher than that of adenosine (adenine riboside, AR, Supplementary material, Figure S5), indicating an inductive effect of the N^6-substituents in the reactivity of the N^6-hydrogen (6-NH group) (Steenken 1989). Differently from CKs (Brizzolari et al. 2016), all four CKS showed a similar activity that could be explained by the presence of the N^9-ribose moiety (a withdrawing group according to Steenken 1989), able to counteract ("neutralize") the electron-donating effect of N^6-substituents.

Among CKs and CKRs, only iPAR was able to scavenge *in vitro* the $O_2^{\bullet-}$ radicals (Supplementary material, Figure S7) although this activity was lower than that of caffeic acid, a well-known inhibitor of superoxide anions (Gülçin 2006). These results are similar to those recently obtained by means of cellular assays by Dassano et al. (2014) suggesting that part of biological activity of CKs and CKRs may be associated to the structure-related antioxidant capacity of the compounds.

2.3. Scavenger activity of CKs and CKRs against lipid peroxyl radicals ($LP\text{-}OO^{\bullet}$)

Results for the *in vitro* scavenging activity of CKs and CKRs against lipid peroxyl radicals ($LP\text{-}OO^{\bullet}$) show that only *p*-T and *p*-TR were weak scavengers (concentration range 0.18 mM–3.6 Mm). At concentrations below 1 mM, a somewhat stronger LP inhibition effect of *p*-TR in comparison with *p*-T can be observed (Supplementary material, Figure S6).

This result suggests that the role of cytokinins in plant lipoperoxidation observed by some autors (Wang et al. 2003; Stoparić and Maksimović 2008) is due more to some regulatory effect on oxidative stress in cells than to an intrinsic capability of the molecules to react with the lipoperoxyl radicals.

3. Experimental

A detailed description, including Tables with calculated IC50 (not listed in the text) is provided in the supplementary material.

4. Conclusions

A few, representative cytokinin ribosides (CKRs, **2a–d**) were evaluated by ORAC and TEAC assays in order to examine their attitude to react with synthetic radicals according to HAT or ET mechanisms. The DPPH assay (mixed HAT/ET) showed no reaction of CKs and CKRs in our experimental conditions.

In vitro scavenging activity of chemically generated, biologically relevant radicals was examined and only a week inhibitory activity of *p*-T and *p*-TR against lipoperoxyl radicals (LP-OO$^{\bullet}$) could be detected whereas OH radical showed a similar reactivity with all CKRs and the superoxide radical $O_2^{\bullet -}$ reacted only with iPAR.

Our results should be integrated by cellular assays in order to show the effect of CKRs (and CKs) on the biological regulators of the oxidative stress.

Disclosure statement

No potential conflict of interests were reported by the authors.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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