Multiple roles for Vitamin B₆ in plant acclimation to UV-B

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Direct and indirect roles of vitamin B₆ in leaf acclimation to supplementary UV-B radiation are shown in vitamin B₆ deficient Arabidopsis thaliana mutant rsr4-1 and C24 wild type. Responses to 4 days of 3.9 kJ m⁻² d⁻¹ biologically effective UV-B dose were compared in terms of leaf photochemistry, vitamer content, and antioxidant enzyme activities; complemented with a comprehensive study of vitamer ROS scavenging capacities. Under UV-B, rsr4-1 leaves lost more (34%) photochemical yield than C24 plants (24%). In the absence of UV-B, rsr4-1 leaves contained markedly less pyridoxal-5′-phosphate (PLP) than C24 ones, but levels increased up to the C24 contents in response to UV-B. Activities of class-III ascorbate and glutathione peroxidases increased in C24 leaves upon the UV-B treatment but not in the rsr4-1 mutant. SOD activities remained the same in C24 but decreased by more than 50% in rsr4-1 under UV-B. Although PLP was shown to be an excellent antioxidant in vitro, our results suggest that the UV-B protective role of B₆ vitamers is realized indirectly, via supporting peroxidase defence rather than by direct ROS scavenging. We hypothesize that the two defence pathways are linked through the PLP-dependent biosynthesis of cystein and heme, affecting peroxidases.

Vitamin B₆ (pyridoxine, PN) and its vitamer derivatives pyridoxal (PL), pyridoxamine (PM) and its phosphorylated analogues have dual roles in plants. They are important for both development¹⁻⁴ and stress tolerance⁵⁻¹⁰. The role in development is likely to be due to the fact that the pyridoxine vitamer pyridoxal 5′-phosphate (PLP) is a crucial co-factor of a range of enzymes important for biosynthesis of building blocks of biological macromolecules⁵⁻⁶. On the other hand, one vital mechanism behind the stress tolerance conferred by the pyridoxine vitamers is their ability to function as quenchers of reactive oxygen species⁷⁻¹⁰.

Vitamin B₆, primarily PLP, is in plants, fungi and most eubacteria synthesized from ribose 5-phosphate, glyceraldehyde 3-phosphate and glutamine by a large 24 polypeptide multisubunit complex (as revealed from studies of the Bacillus subtilis enzyme) that consists of 12 units of the PDX1 synthase protein and 12 units of the PDX2 glutaminase protein¹⁰. In plants, there is one gene encoding PDX2, the mutation of which is lethal, and three PDX1 genes (PDX1.1-PDX1.3). PDX1.1 and PDX1.3 are enzymatically functional proteins. PDX1.2 on the other hand is not catalytically active. Instead, this protein has a regulatory role on vitamer biosynthesis through interaction with the multisubunit enzyme, primarily during stress⁵⁻⁶. Environmental factors that have shown to cause stress in A. thaliana plants that have been mutated in genes of one of the PDX1 subunits include salt and osmotic stress¹³, high light and photo-oxidative stress¹⁴⁻¹⁶, heat¹⁴⁻¹⁶, and ultraviolet-B light (UV-B, 280–315 nm)¹⁷⁻¹⁸.

UV-B is part of the radiation spectrum of the sun that plants are exposed to and dependent on. UV-B is generally a morphological factor under normal conditions¹⁷⁻¹⁹ but can be a stressor in extreme environments or unusual circumstances²⁰⁻²¹, or when plants are exposed to multiple stresses at the same time²². UV-B is generally sensed by plants through the UV RESISTANCE LOCUS 8 (UVR8) photoreceptor and its downstream signaling components²³ which in turn regulate over 100 genes. The gene encoding the PDX1.3 protein is one of these and is up-regulated by UV-B²²⁻²⁵. Also, UV-B exposure leads to increased levels of PDX1 protein in Arabidopsis²⁶⁻²⁷ and increased levels of vitamin B₆⁶. In fact, one of the more non-specific modes of action of UV-B on plants is the formation of ROS²⁸ and the UV-B-induced increase in vitamin B₆ content in plants most likely is a result of increased oxidative pressure. This oxidative pressure was shown to increase in an A. thaliana pdx1.3 mutant that still had a functional PDX1.1 gene.

In order to further elucidate the roles of the different pyridoxine vitamers in planta, C24 wild type and rsr4-1 mutant A. thaliana were used to draw conclusions about the roles that ROS and the pyridoxine vitamers play during UV-B exposure. Created from C24 using the mutagenic alkylating agent ethyl-methanesulfonate, a glabrous,
Supplemental UV imposed a mild stress in both genotypes, as indicated by lower maximum (Fv/Fm) and regulated (Y(NPQ)) PSII photochemical yields than in controls (Table 1). The UV-induced loss was higher (ca. 32%) in the rsr4-1 mutant than in the C24 wild type (ca. 22%). Yields of photochemical energy conversion in PSII were lower either entirely (C24 plants) or mostly (rsr4-1 plants) at the expense of increasing energy dissipation via non-regulated non-photochemical quenching (Y(NO)), signifying suboptimal capacities of photoprotective reactions, that may lead to photodamage. The UV treatment had no effect on the quantum yield of regulated non-photochemical quenching (Y(NPQ)) in C24 leaves but resulted in a 9% increase in the rsr4-1 mutant. The latter change brought up Y(NPQ) in UV-treated rsr4-1 to values similar to those in C24. Such an increase indicates elevation of defence against photo-oxidative stress at the PSII level in the mutant that is not occurring in the wild type. This might be explained by the above-mentioned xanthophyll-cycle related changes observed in the pdx1 mutant under high light stress. However, this pathway is probably inefficient in the rsr4-1, as indicated by the relatively small extent of its increase compared to that of Y(NO). Although the biochemical explanation of non-regulated non-photochemical pathways is still incomplete, it is generally agreed that some of the multiple pathways behind Y(NO), especially those driving this parameter above 0.2–0.25 in light acclimated leaves, reflect the inability of a plant to protect itself against damage caused by excess illumination, presumably via increased ROS production. In the following, we examined how the antioxidant systems of C24 and rsr4-1 leaves met this challenge.

### Vitamin B6 content of A. thaliana leaves, and in vitro ROS neutralizing potential of these compounds.

The three basic vitamins (PL, PM, PN) and the activated form of vitamin B6 (PLP) were quantified in C24 wild type and rsr4-1 mutant leaves using HPLC. PN contents were low in both genotypes, representing only 1–2% of the total, and showing that PN is the least common B6 vitamer in both genotypes (Table 2). In C24

| Treatment | Fv/Fm | φPSII | Y(NPQ) | Y(NO) |
|-----------|-------|--------|--------|--------|
| C24 UV    | 0.855 | 0.655  | 0.123  | 0.221  |
| rsr4-1 C  | 0.853 | 0.658  | 0.114  | 0.201  |
| rsr4-1 UV | 0.574 | 0.437  | 0.124  | 0.440  |

**Table 1.** Effects of supplemental UV radiation on maximum (Fv/Fm) and 55 μmol m⁻² s⁻¹ PAR acclimated effective (φPSII) quantum yields, the regulated (Y(NPQ)) and non-regulated (Y(NO)) non-photochemical quenching of PSII (Materials and Methods). Data are presented as means ± SD. *UV effect: significant difference between control (C) and UV-exposed (UV) leaves (p < 0.05, n = 8) of the same genotype. #Genotype effect: significant difference between wild type (C24) and B6 mutant (rsr4-1) control leaves (p < 0.05, n = 8) under the same irradiation conditions.
leaves, the amounts of PL and PLP were the highest, while PLP concentration in the mutant were below the one in the wild type (Table 2). Our data are in agreement with those of Wagner et al.: they established a PL > PM > PN order of the amounts of these compounds in both genotypes. They also found a higher PL/PM ratio in C24 than in rsr4-1 leaves, although their absolute concentrations were different from ours, most likely due to differences in growth conditions and extraction procedures. Our observations do not agree with Havaux et al. who found high PN and PM, and low PL concentrations. However, these authors analysed chloroplasts only (as opposed to whole leaf extracts in our case) and used a different Arabidopsis wild type, Col-0. The UV treatment applied led to elevated concentrations of all vitamins in mutant leaves but did not affect the B6 content in the wild type. The UV treatment brought the PLP levels in rsr4-1 leaves up to those in C24 under UV and increased the PL and PM contents of the mutant to 30% higher than those in the wild type (Table 2).

In vitro analyses of vitamer reactivities toward ROS provide an excellent tool to study their antioxidant potential. However, the realization of these studies in planta depends on a number of factors, primarily on vitamer localization. Several studies of vitamin B6 quenching of ROS have been previously performed but these investigations either were limited to one or two ROS only or involved fewer forms of vitamin B6 than the present work. Thus, to our best knowledge, the results in Table 3 provide the first comprehensive data set of antioxidant capacities of four B6 vitamers (the three basic forms PL, PM, PN, and the activated form PLP) against the four principal ROS: singlet oxygen (1O2), superoxide anion (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (•OH) antioxidant abilities were expressed as µM vitamer/µM Trolox equivalents. ND, non-detectable. Data represent means ± SD (n = 6–8).

Table 3. ROS-specific neutralizing capacities of B6 vitamers. Singlet oxygen (1O2), superoxide anion (O2•−), hydrogen peroxide (H2O2) and hydroxyl radical (•OH) antioxidant abilities were expressed as µM vitamer/µM Trolox equivalents. ND, non-detectable. Data represent means ± SD (n = 6–8).

|                | anti-1O2 | anti-O2•− | anti-H2O2 | anti-•OH |
|----------------|----------|-----------|-----------|----------|
| pyridoxal (PL) | 69.81 ± 6.84 | 14.47 ± 5.30 | 25.04 ± 2.78 | 42.71 ± 0.54 |
| pyridoxamine (PM) | 459.7 ± 52.19 | 0.5 ± 0.09 | 39.69 ± 7.20 | 16.21 ± 0.58 |
| pyridoxine (PN) | 5.69 ± 0.06 | 0.47 ± 0.06 | 48.17 ± 10.09 | 8.29 ± 0.65 |
| pyridoxal 5′-phosphate (PLP) | ND | 1.04 ± 0.02 | 287.90 ± 64.81 | 66.71 ± 4.45 |

Leaf antioxidant responses to supplemental UV-B. 

In the following, we compare the activities of enzymes neutralizing electron transfer derived ROS (H2O2 and O2•−) in leaves of wild type and mutant plants and discuss their UV-B-induced changes. Because •OH neutralization is only supported non-enzymatically, this was also included in the analysis.

Under growth light conditions, in the absence of UV, the rsr4-1 mutant controlled cellular H2O2 concentrations by significantly higher catalase (CAT, EC 1.11.1.6) activity, while keeping class III peroxidase (POD, EC 1.11.1.7)

|                |                  |                  |            |            |
|----------------|------------------|------------------|------------|------------|
|                | pyridoxal (PL)   | pyridoxamine (PM)| pyridoxine (PN) | pyridoxal 5′-phosphate (PLP) |
| C24 C          | 1.048 ± 0.132    | 0.694 ± 0.060    | 0.064 ± 0.037 | 1.357 ± 0.277 |
| C24 UV         | 1.160 ± 0.177    | 0.690 ± 0.138    | 0.052 ± 0.015 | 1.184 ± 0.251 |
| rsr4-1 C       | 1.117 ± 0.147    | 0.704 ± 0.070    | 0.030 ± 0.008 | 0.797 ± 0.207* |
| rsr4-1 UV      | 1.530 ± 0.141**  | 0.895 ± 0.103**  | 0.062 ± 0.009** | 1.088 ± 0.164* |

Table 2. B6 vitamer profiles of A. thaliana leaves. Means ± SD are expressed as ng vitamer mg−1 leaf FW. *UV effect: significant difference between control (C) and UV-exposed (UV) leaves (p < 0.05, n = 8) of the same genotype. **Genotype effect: significant difference between wild type (C24) and B6 mutant (rsr4-1) control leaves (p < 0.05, n = 8) under the same irradiation conditions.
activity lower than the wild type C24 plants. There were no significant differences between the two genotypes in superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), or glutathione peroxidase (GPX, EC 1.11.1.9) activities (Fig. 1). In response to the UV treatment, POD, APX and GPX activities increased in wild type C24 leaves whereas SOD and CAT remained unchanged. Unchanged SOD and increased peroxidase activities under UV are in line with our observation of higher activation of APX and POD than of SOD in tobacco leaves. This strategy keeps cellular H2O2 concentrations low in order to decrease the risk of oxidative damage, potentially aggravated by the UV-B photo-conversion of H2O2 to •OH. The increased •OH scavenging capacity in C24 leaves under UV-B (Fig. 1), that was also found in tobacco leaves, may serve as a second line of defence. In contrast, rrs4-1 leaves were unable to up-regulate any peroxidase (GPX, APX or POD) activities upon the UV treatment. The observed strong decrease in SOD activity of UV-treated rrs4-1 plants lowers the production of H2O2 via O2•− dismutation and highlights the lack of efficient direct H2O2 scavenging in the mutant.

The lack of change in CAT activity in UV-irradiated C24 plants (Fig. 1) suggests that peroxisomal H2O2 production is either low or well regulated already and does not initiate oxidative stress. Although UV irradiation
of wheat under different experimental conditions upregulated CAT39,40, we found no significant change in CAT activity in UV experiments with tobacco leaves either (Czégény et al., unpublished). Our results may be explained by assuming (i) a low photorespiratory activity in wild type plants grown under relatively low PAR in our growth chambers, and/or (ii) catalase reactions being unaffected by the applied UV treatment. Either hypothesis is in line with the lack of CAT UV response in the rsr4-1 mutant (Fig. 1).

A weak line of enzymatic H$_2$O$_2$ neutralization explains the need for more efficient $\cdot$OH scavenging in the vitamin B$_6$ mutant. Accordingly, non-treated rsr4-1 plants had 40% more efficient $\cdot$OH neutralizing capacity than the wild type and both genotypes were capable of upregulating this activity under UV. Although B$_6$ vitamers, especially PLP, are efficient $\cdot$OH antioxidants in vitro (Table 3), differences in leaf $\cdot$OH antioxidant capacity do not match the vitamin B$_6$ content, and thus a strong contribution of other antioxidants has to be assumed. Potential other $\cdot$OH antioxidants include α-tocopherol, phenolic compounds, ascorbate and reduced glutathione (GSH)41–44, each shown to increase under mild UV-B exposure45. Among the above candidates, both the plant phenol chlorogenic acid and α-tocopherol were found to be more reactive to $\cdot$OH than ascorbate or GSH43. Chlorogenic acid is synthesized in A. thaliana46 and, similarly to other phenolic compounds, its synthesis is regulated by the UVR8 photoreceptor47.

Figure 2 is a schematic representation of pathways discussed in the present work. UV-B inducible and antioxidative routes are shown in blue and red, respectively. Routes are discussed in the text according to the circled identifying numbers.

Figure 2. A schematic representation of pathways discussed in the present work. UV-B inducible and antioxidative routes are shown in blue and red, respectively. Routes are discussed in the text according to the encircled identifying numbers.
Methods

Plant growth and UV treatment.  *A. thaliana* plants (C24 wild type and *rsr4-1* mutant) were grown using 90 μmol m⁻² s⁻¹ photosynthetically active radiation (PAR) in growth chambers with constant 70% relative humidity and 6 h/18 h, 22°C/18°C day/night conditions. 5-week-old plants were divided into two groups, each containing eight plants from each genotype. The first group (UV plants) was exposed to supplemental UV radiation from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) through a cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) between 10.00 and 14.00 daily for 4 days. The UV spectrum centred around 318 nm. UV-B irradiation corresponded to 3.9 kJ m⁻² d⁻¹ biologically effective dose [43]. The second group (control plants) were kept under PAR only. Photosynthesis measurements were carried out at the end of the 4-day treatments, and then leaves were frozen in liquid N₂ and stored at −80°C for analytical measurements.

Chlorophyll fluorescence measurements.  Photosynthesis was characterized by chlorophyll fluorescence measurements using the MAXI version of Walz Imaging PAM. Maximum quantum yield of photosystem II (Fv/Fm) was measured after 30 min dark adaptation. Light acclimated effective PSI quantum yield (φPSII), regulated non-photochemical quenching (Y(NPQ)) and non-regulated non-photochemical quenching (Y(NO)) were determined under 55 μmol m⁻² s⁻¹ blue actinic light according to Klímkahm & Schreiber [31]. In this model, the three PSI quantum yields are complementary as φPSII + Y(NPQ) + Y(NO) = 1, representing three possible pathways of disposing quanta.

HPLC analysis.  Thirty mg of leaves were ground in liquid nitrogen, placed into a plastic tube and extracted with 1 ml of 50 mM H₃PO₄ solution using ultrasonic bath for 15 min. The resulting suspension was centrifuged at 20,660 × g. Supernatants were filtered using a 0.22 μm PTFE syringe filter and analysed by high-performance liquid chromatography (HPLC). HPLC-FLD analysis was performed using a PerkinElmer Series 200 HPLC system consisting of a vacuum degassing unit, quaternary pump, autosampler, column thermostat and a fluorescence detector (FLD). Separations were achieved by using a Phenomenex Synergi 4 µm Hydro-RP 80 Å, 250 × 4.6 mm column. Column temperature was maintained at 25°C. For elution, 50 mM H₃PO₄ (eluent A) according to Lawrence & Burk [60]. The reaction mixture contained 1 mM EDTA, 0.2 mM NADPH, 1 mM NaN₃, 1 mM reduced glutathione and 1 U mL⁻¹ glutathione reductase in 50 mM potassium phosphate buffer (pH 7.0) and the reaction was started by adding 0.25 mM H₂O₂. Following this, absorbance at 340 nm was measured (every second) for 4 min. Enzyme activity was determined as U GPX g⁻¹ protein.

Antioxidant measurements on leaf samples.  Superoxide dismutase (SOD, EC 1.15.1.1) activity measurements were carried out as described earlier [45], based on the inhibition of nitroblue tetrazolium (NBT) reduction by xanthine - xanthine-oxidase generated superoxide anions, and results were expressed as U SOD mg⁻¹ protein.

Class III peroxidase (POD, EC 1.11.1.7) activity was measured via the oxidation of ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) [46] that was found to be the most effective general POD substrate for UV-treated samples [37]. The colour change was detected by Multiskan FC plate reader (Thermo Fischer Scientific, Shanghai, China) at 651 nm and POD activities were given as U POD mg⁻¹ protein.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined by following NADPH oxidation at 340 nm according to Lawrence & Burk [60]. The reaction mixture contained 1 mM EDTA, 0.2 mM NADPH, 1 mM NaN₃, 1 mM reduced glutathione and 1 U mL⁻¹ glutathione reductase in 50 mM potassium phosphate buffer (pH 7.0) and the reaction was started by adding 0.25 mM H₂O₂. Following this, absorbance at 340 nm was measured (every second) for 4 min. Enzyme activity was determined as U GPX g⁻¹ protein.

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Ascorbate peroxidase (APX, EC 1.11.1.11) activities were measured according to Nakano & Asada [47], following ascorbate oxidation as decrease in absorbance at 295 nm with a spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Tokyo, Japan). The reagent solution contained 0.5 mM ascorbic acid, 1 mM H₂O₂, and 1 mM EDTA in a Na-phosphate buffer (50 mM, pH 7.0) plus leaf samples. Values were corrected for the APX independent, direct oxidation of H₂O₂. Enzyme activities were described as U APX mg⁻¹ protein.

Catalase (CAT, EC 1.11.1.6) activity was measured as described in Aebi et al. [51], by following the decrease in H₂O₂ concentration as 240 nm absorbance for 70 seconds (measured at every second). The assay contained 18.6 mM H₂O₂ and 1 mM EDTA in 50 mM Na-phosphate buffer (pH 7.0) and the reaction was started by adding the leaf sample. Activity was expressed as U CAT mg⁻¹ protein.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined by following NADPH oxidation at 340 nm according to Lawrence & Burk [60]. The reaction mixture contained 1 mM EDTA, 0.2 mM NADPH, 1 mM NaN₃, 1 mM reduced glutathione and 1 U mL⁻¹ glutathione reductase in 50 mM potassium phosphate buffer (pH 7.0) and the reaction was started by adding 0.25 mM H₂O₂. Following this, absorbance at 340 nm was measured (every second) for 4 min. Enzyme activity was determined as U GPX g⁻¹ protein.

Hydroxyl radical (*OH) scavenging capacity was assessed via measuring the inhibition of the oxidation of terephthalic acid (TPA) to fluorescent hydroxyterephthalate (HTPA) by *OH from the Fenton reaction [61] in a Hitachi F-7000 spectropho-photometer (Hitachi High-Technologies, Tokyo, Japan) with excitation at 315 nm and emission at 420 nm. The method is based on the fact that the antioxidant containing leaf samples can delay the *OH-driven formation of HTPA. Hydroxyl radical antioxidant capacities were characterized by the amounts of plant samples needed to decrease HTPA fluorescence by 50% [46] and were given as μM Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent g⁻¹ leaf fresh weight.
ROS specific antioxidant capacities of vitamin B<sub>6</sub>. Singlet oxygen (1O2) scavenging was determined by the ability of B<sub>6</sub> vitamers to decrease the oxidation of DPBF (1,3-diarylisobenzofuran) by 1O2. As the 1O2 source we used methylene blue (MB) that was irradiated with 50 μmol m<sup>−2</sup> s<sup>−1</sup> red light (600–650 nm) for 1 minute. 1 mL of reaction mixture contained 20 μM MB and 100 μM DPBF in 60:40 v/v methanol/water. Oxidation of DPBF caused a decrease in absorbance which was followed at 410 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). 1O2 scavenging abilities of B<sub>6</sub> vitamers were assessed based on their ability to lessen the decrease of 410 nm absorbance and were presented as μM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents.

Superoxide anion (O<sup>2−</sup>) scavenging ability of vitamin B<sub>6</sub> was characterized via a slightly modified assay described by Majer et al. B<sub>6</sub> vitamers can inhibit the superoxide-induced reduction of NBT (nitro blue tetrazolium) to formazan. The reaction mixture contained 0.3 mM xanthine, 0.3 mM EDTA in 50 mM K-phosphate buffer (pH 7.2) and the reaction was started by adding 0.015 U xanthine oxidase. Formazan production was measured as absorbance change at 540 nm in a plate reader. Results were expressed as μM Trolox equivalents.

Hydroxyl radical (*OH) scavenging capacities were measured using a modification of the TBA-RS (thiobarbituric acid reactive substances) assay. Such an indirect approach was necessary, because all B<sub>6</sub> vitamers fluoresce upon UV excitation preventing the use of the more direct, terephthalate based method that was applied for characterizing leaf extracts. The assay contained 0.1 mM FeSO<sub>4</sub>, 1 mM EDTA, 0.25 mM ascorbate, 1 mM H<sub>2</sub>O<sub>2</sub>, and 3 mM deoxyribose in 20 mM potassium-phosphate buffer (pH 7.4). Hydroxyl radicals generated in a Fenton reaction oxidize deoxyribose yielding products that form a pink chromogen upon incubation with 0.1 w/v% thiobarbituric acid at 40 °C for 30 min in an 8% TCA solution. Added B<sub>6</sub> vitamers competed with deoxyribose for the hydroxyl radicals and thus decreased chromogen formation that was followed as 540 nm absorbance using a plate reader. Results were expressed as μM Trolox equivalents.

Statistics. All treatment groups contained 8 plants of each genotype as biological replicates that were assayed separately to form one (n = 8) data set. ROS reactivity measurements of pure vitamers, where no leaf material was used, were carried out with 6–8 technical repetitions to calculate one mean value. For each variable, differences between means were compared with two-sample Student’s t-tests and significantly different (p < 0.05) means are marked with either different letters (in graphs) or different symbols (in tables).

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**Author Contributions**

E.H. and Å.S. conceived the research plan with contributions of Gy.Cz.; Gy.Cz. and E.H. designed the experiments; Gy.Cz. performed most of the experiments with the exception of HPLC analysis that was done by L.K.; the article was written with contributions of all the authors.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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