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Structure–activity relationships of *N*-phenyl-*N*′-benzothiazol-6-ylurea synthetic derivatives: Cytokinin-like activity and adventitious rooting enhancement

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ABSTRACT

Some years ago we demonstrated the cytokinin-like activity of the synthetic *N*-phenyl-*N*′-benzothiazol-6-ylurea (PBU) and a relevant adventitious rooting adjuvant activity of symmetric urea derivatives devoid of any cytokinin- or auxin-like activity *per se*. Here we report the synthesis and the biological activity evaluation of nine symmetric or asymmetric ureas/thioureas, structurally related to PBU. None of them show cytokinin-like activity, while we demonstrate for the first time that PBU interacts with *Arabidopsis* cytokinin receptor CRE1/AHK4 in a heterologous bioassay system. Among the PBU derivatives, all the symmetric ureas/thioureas show an adventitious rooting adjuvant activity in various bioassays, confirming that this activity is strictly dependent on their chemical structure.

1. Introduction

As a result of competitive researches carried on by the middle of the 1950s, Miller and co-workers demonstrated that the 6-furfurylaminopurine (kinetin) is a growth promoter in plant tissue culture (Miller et al., 1955, 1956), while Shantz and Steward showed that the *N,N*′-diphenylurea (DPU) is able to stimulate cell division in mature carrot phloem cell cultures (Shantz and Steward, 1955). Miller's work gave rise to the discovery of cytokinins, naturally occurring N^6-substituted adenine derivatives containing an isoprenoid or aromatic side chain (Mok and Mok, 2001 and Strnad, 1997, respectively). These plant hormones are involved in many essential developmental processes, such as cell division, senescence, nutrient mobility, chlorophyll biosynthesis, as well as symbiosis, pathogenesis, abiotic stress response (Mok and Mok, 2001; Werner et al., 2001; Haberer and Kieber, 2002; Werner and Schmülling, 2009). On the other hand, although it has been later demonstrated that DPU is a rather weak cytokinin-like synthetic compound (Miller, 1960; Bottomley et al., 1963), the fortuitous finding of DPU activity was the starting point for the discovery of much more potent urea derivatives, as the *N*-phenyl-*N*′-(2-chloro-4-pyridyl)urea (CPPU) and the *N*-phenyl-*N*′-(1,2,3-thiadiazol-5-yl)urea (thidiazuron, TDZ), showing a cytokinin-like activity sometimes exceeding that of the adenine-type derivatives (Takahashi et al., 1978; Mok et al., 1982; Shudo, 1994). Their high cytokinin-like activity was connected to their extreme stability in plant tissue, as it has been demonstrated that the TDZ molecule remains intact in tissue culture systems and that its metabolism, consisting in glucosides formation, is extremely slow (Mok and Mok, 1985). Moreover, it has been demonstrated that they strongly inhibit the activity of cytokinin oxidases, the plant enzymes that catalyse the degradation of adenine-type cytokinins by selectively cleaving unsaturated N^6-isoprenoid side chain (Victor et al., 1999; Mok and Mok, 2001 and references herein). Thus, their high cytokinin-like activity could be the result of indirect effects on the regulation of the endogenous cytokinin metabolism. At the same time, the similarity in the biological activity of these two structurally unrelated classes of compounds, the naturally occurring adenine derivatives and the synthetic ureas, has posed one of the most interesting problems in cytokinin structure–activity relationships, i.e. whether they can work through interaction with a common cellular target, the cytokinin receptor. Finally, three membrane-bound hybrid sensor histidine kinases, CRE1/AHK4, AHK2 and AHK3, have been identified as cytokinin receptors in *Arabidopsis thaliana* (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001). They mediate signal transduction in a two-component

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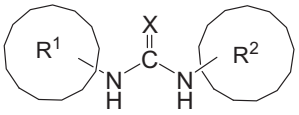
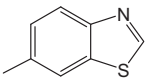
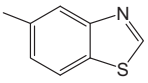
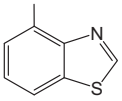
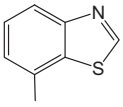
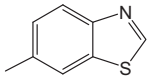
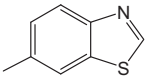
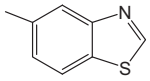
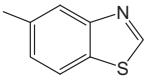
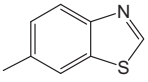
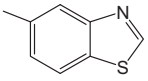
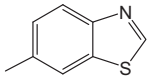
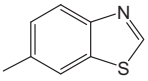
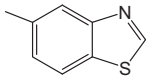
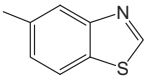
signalling phosphorelay cascade similar to the procariotic one (To and Kieber, 2008) and are all required for cytokinin perception *in planta*, even if they act redundantly (Higuchi et al., 2004; Riefler et al., 2006). Interestingly, by a cytokinin heterologous assay system with microbial cells, it has been demonstrated that TDZ is also able to exert a direct cytokinin activity through the cytokinin receptors (Yamada et al. 2001; Spíchal et al., 2004; Romanov et al., 2006). As result of structure–activity relationship studies of synthetic *N*,*N*′-diaryl substituted ureas, we reported the cytokinin-like activity of *N*-phenyl-*N*′-benzothiazol-6-ylurea (PBU), structurally related to thidiazuron, and the unexpected adventitious rooting adjuvant activity of some symmetric urea derivatives, showing any biological activity *per se* (Ricci and Bertoletti, 2009 and references herein; Rolli and Ricci, 2009; Rolli et al., 2011). With the aim of exploring the dependence of the cytokinin-like activity and the adventitious rooting enhancement showed by the *N*,*N*′-diaryl substituted urea derivatives on their chemical structure, we herein report the synthesis and the biological evaluation of a number of compounds rationally related to PBU and to symmetric ureas previously described by some of us (Ricci et al., 2001a,b). Starting from the lead compound PBU, we synthesized nine ureas (Table 1) designed on the basis of the benzothiazolyl substituent positional isomerism (compounds **1–3**), of the oxygen–sulphur isosteric replacement in the urea skeleton (compounds **6–9)** and on the basis of the *N*,*N*′-symmetric introduction of the benzothiazole substituent (compounds **4, 5** and **8, 9**). The hypothetical cytokinin-like activity of the compounds was analysed by different bioassays based on various biological effects of cytokinins: the regeneration of tomato cotyledon explants and the *Amaranthus* bioassay. As ultimate test to corroborate a direct cytokinin-like activity, we investigated the ability of test compounds to interact with the *Arabidopsis* cytokinin receptor CRE1/AHK4. On the other hand, their effect on adventitious root formation was analysed by adventitious rooting of *Arabidopsis* etiolated hypocotyls. Further, three more adventitious rooting assays (*Vigna radiata* hypocotyl, apple cutting and apple slice bioassays) were performed only with the compounds that positively affected the previous *Arabidopsis* etiolated hypocotyl bioassay.

Table 1
Structures of the compounds.



Compound	X	R^1	R^2
PBU	O		
1	O		
2	O		
3	O		
4	O		
5	O		
6	S		
7	S		
8	S		
9	S		

2. Results and discussion

2.1. Chemistry

Compounds **1**–**9** and PBU (Table 1) were prepared by a three-step procedure starting from the commercial suitable chloronitroanilines that, refluxed with formic acid to give the formyl derivatives, upon following heterocyclization in the presence of sodium sulphide, afforded the target nitrobenzothiazoles, previously reported by Vel'tnan, 1960. The only 6-nitrobenzothiazole was more conveniently synthesized through direct nitration of benzothiazole, commercially available, following the procedure of Gallagher et al. (1980). The obtained nitrobenzothiazoles were then reduced by iron in acetic acid, affording in good yields the corresponding amines. The aminobenzothiazoles, described elsewhere (Spieler and Prijs, 1950; Shimidzu and Uno, 1973; Gallagher et al., 1980) were finally heated with phenylisocyanate, BOC anhydride or phenylisothiocyanate to afford the target ureas and thioureas (Fig. 1). *N*-phenyl-*N*′-benzothiazol-5-ylurea (**1**) and thioureas **6** and **7** have already been described (El-Kerdawy et al., 1999; Chakrabarty et al., 2010, respectively) and the data of the compounds synthesized for the present study were consistent with the characterization literature data. The new compounds **2**–**5** and **8**–**9** were characterized by elemental analyses and NMR spectral data. These data, detailed in the experimental part, are consistent with the suggested structures.

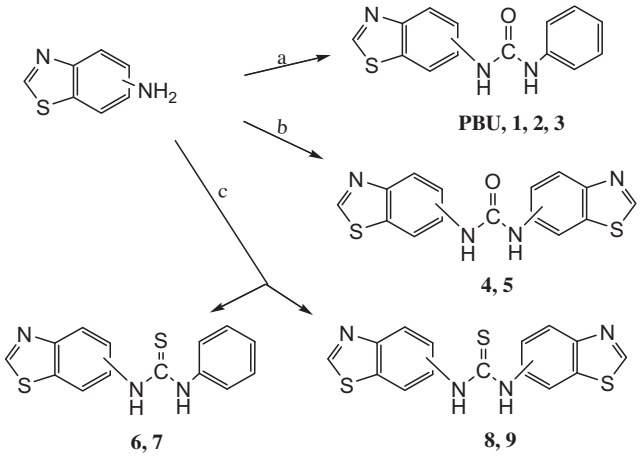


Fig. 1. Scheme of synthesis; reagents and conditions: (a) PhNCO, $PhCH_3$, reflux 1 h, (b) di-*t*-butyl dicarbonate (BOC_2O), pyridine, 110 °C (sealed vessel) 18 h, (c) PhNCS, $PhCH_3$, reflux 6 h.

2.2. Activity in cytokinin bioassays

Taking advantage of the tomato explant regeneration assay, the cytokinin-like activity of PBU has been already reported (Ricci et al., 2001b), thus the same bioassay was used to compare the biological activity of its derivatives. Then, all the test compounds were tested in another classical cytokinin bioassay, the *Amaranthus* one. These two bioassays have been developed based on the various biological effects of cytokinins, i.e. to assess the induction of organogenesis with a long assay time and the stimulation of a biochemical event with a short assay time, respectively. Finally, the capacity of the test compounds to interact with the *Arabidopsis* cytokinin receptor CRE1/AHK4 was analysed in the heterologous bacterial assay. This was the only one cytokinin receptor we used in this study rather than either of the other two *Arabidopsis* cytokinin receptors, AHK2 and AHK3, because it has been reported that CRE1/AHK4 provokes a severalfold higher response in *Escherichia coli* than AHK3, even if they showed different ligand specificity (Spíchal et al., 2004). It has also been demonstrated that in planta transmission of a cytokinin signal through AHK3 and AHK2 was several fold lower than through CRE1/AHK4 (Hwang and Sheen, 2001). None of the PBU derivatives showed any cytokinin-like activity in these bioassays (data not shown), suggesting that only *N*-phenyl-*N*′-benzothiazol-6-ylurea (PBU) possesses the desired chemical features for the tested activity. All the changes, based on positional isomerism, O = S isosterism and symmetry, introduced in the designed analogues **1–9,** have completely cancelled the cytokinin-like activity related to PBU. The lacking of activity obtained by the thiourea derivatives was the only result that we could expect, as it has already been reported that HNCSNH bridge lowers the biological activity of urea derivatives (Bruce and Zwar, 1966). Strangely, also PBU was inactive in the *Amaranthus* assay, confirming the different sensitivity of the bioassays, even if stable synthetic cytokinins should display similar activities in different bioassays (Mok, 1994). On the other hand, in the heterologous bacterial assay, PBU was able to directly interact with the *Arabidopsis* cytokinin receptor CRE1/AHK4, as it was possible to detect the β-galactosidase activity in a concentration dependent manner. PBU exerted its activity to a lower extent and at concentration 100-fold higher (100 μM) than that required for receptor activation by TDZ (Fig. 2). Thus, from these results, we can infer that PBU is not a very good ligand of this cytokinin receptor, as the weak direct response, significantly different from that of DMSO used as control solvent, is induced only at the highest concentration tested, but

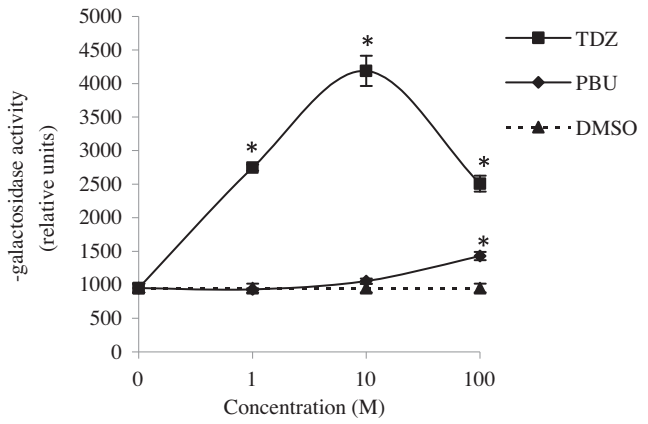


Fig. 2. Activation of cytokinin receptor CRE1/AHK4 by PBU. β-galactosidase activity is expressed as Miller units by the formula $1000 \times [OD_{420}/(OD_{600} \times 20\ \mu l \times 120\ min)]$.* The difference between each mean and the control (DMSO) is significantly different, Student's *t*-test, $p < 0.05$. Error bars show SE ($n = 6$).

once more this is not a strange behaviour. In fact, it has been recently reported that some kinetin derivatives, highly active in cytokinin bioassays, are not able to activate CRE1/AHK4 (Mik et al., 2011). However, as the PBU cytokinin-like activity previously reported (Ricci et al., 2001b; Carra et al., 2006; Torelli et al., 2006; Rolli et al., 2011) and its slight CRE1/AHK4 activation, here demonstrated for the first time, seem inconsistent results, an indirect effect through a perturbation of the endogenous cytokinin metabolism cannot be excluded. A hypothetical mode of action could be the interference with the N- or O-glucosylation of endogenous cytokinins as well as the inhibition of the cytokinin oxidase, as previously reported for other urea derivatives (Mok et al., 2005; Kopečný et al., 2010).

2.3. Activity in adventitious rooting bioassays

Adventitious rooting is a postembryonic organogenic process in which new root meristems are induced from a regulated sequence of cell proliferation and differentiation at position where roots do not normally originate. A new hormonal balance is required to enable certain somatic differentiated cells, retaining an intrinsic competence to form adventitious roots, to switch their fate into multipotent cells by remarkable changes in gene expression pattern (Busov et al., 2009; Ludwig-Müller, 2009; Abarca and Diaz-Sala, 2009). Auxins are the main inducers of adventitious rooting in plants and their role in the process has been widely studied (Pop et al., 2011 and references herein), whereas cytokinins, usually considered auxin-antagonists, are described as negative regulators of root meristem activity and of lateral root formation (Werner et al., 2003; Werner and Schmülling, 2009). As to the adventitious rooting process, their obvious effect is inhibition and it has been reported that TDZ and 6-benzylaminopurine (BAP) are the most effective (De Klerk et al., 2001). On the other hand, the same Authors demonstrated that the inhibiting effect is strictly related to the strength of the cytokinin, as "weak" cytokinins may even stimulate adventitious rooting in a concentration dependent manner. In fact, during the early stages of the adventitious rooting process, cytokinins may stimulate initial cell divisions that are required to achieve adventitious roots; afterwards, they become inhibitory (De Klerk et al., 2001). Keeping in mind the results obtained in the above mentioned cytokinin bioassays and according to the previously reported capacity of some urea derivatives to enhance the adventitious root formation depending on their chemical structure even without showing any cytokinin-like activity (Ricci and Bertoletti, 2009 and references herein), we decided to analyse the effect of PBU and its derivatives on adventitious rooting. Firstly, using a simple, fast and reliable bioassay, we screened their capacity to enhance adventitious root formation in *Arabidopsis* etiolated hypocotyls (Sorin et al., 2005). In the absence of exogenous auxin, PBU, **1**–**3**, **6** and **7** were completely ineffective in enhancing adventitious root formation, while the symmetric compounds **4, 5, 8** and **9** were differently able to interact with endogenous auxin (Table 2). In fact, compound **4** significantly enhanced the mean adventitious root number at all the concentrations tested, compounds **5** and **8** were ineffective and compound **9** significantly enhanced the mean adventitious root number at 4 and 16 μM. In the simultaneous presence of 0.1 μM indole-3-butyric acid (IBA), as exogenous auxin, the asymmetric compounds **1**–**3**, **6** and **7** were once more completely ineffective. On the contrary, PBU behaved as a "weak" cytokinin-like compound, significantly enhancing the mean adventitious root number at 4 μM and significantly inhibiting it at the highest concentration (16 μM). Thus, it was confirmed that "weak" adenine- and urea-type cytokinins promote adventitious root formation at relatively low concentrations whereas they inhibit the process at supraoptimal concentrations (De Klerk et al., 2001; Ricci

Table 2
Effect of selected compounds on adventitious rooting.

Compound	*Arabidopsis* bioassay[a,b] Comp. concentration (μM)						Mung bean bioassay[a,c] Comp. concentration (μM)				Apple slice bioassay[a,d] Comp. concentration (μM)		
	1		4		16		0.01	0.1	1	10	1	4	16
		+IBA		+IBA		+IBA							
PBU	−2.8	16.1	13.1	29.9*	−6.9	−19.5*	-[e]	-	-	-	9.1	45.4*	−63.6*
4	40.8*	17.9	40.8*	75.3*	63.5*	108.8*	-	-	-	-	-	-	-
5	0.3	12.3	−9.6	41.0*	5.3	34.4*	-	-	-	-	61.0*	42.1*	27.8*
8	−10.8	−2.1	−8.7	58.4*	9.6	103.6*	-	-	-	-	9.5	52.1*	51.8*
9	19.7	41.2	50.2*	43.5*	58.6*	51.3*	19.5*	40.7*	−27.9	−27.1	−0.9	52.5*	15.2*

[a] The results are expressed as a percentage of the control by the formula $[(T - C/C) \times 100]$.
[b] Effect on adventitious rooting of *Arabidopsis* hypocotyls in the absence or in the simultaneous presence (+IBA) of 0.1 μM IBA. The results are expressed as a percentage of the control (hormone free or 0.1 μM IBA, respectively).
[c] Effect on adventitious rooting of *Vigna radiata* hypocotyls.
[d] Effect on adventitious rooting of apple slices in the simultaneous presence of 1 μM IBA.
[e] Ineffective.
* The difference between each mean and the control is significantly different, Student's *t*-test, $p < 0.05$.

et al., 2005). All the symmetric compounds, both ureas **4** and **5**, and thioureas **8** and **9**, significantly enhanced the mean adventitious root number to a different extent when supplemented at 4 and 16 μM. According to the results obtained, only the compounds that enhanced the formation of adventitious roots in *Arabidopsis* etiolated hypocotyls, the symmetric ones and PBU, were used in the following bioassays: rooting of *V. radiata* hypocotyls, rooting of apple cuttings and rooting of apple slices. Compound **9** was able to enhance the adventitious root formation in *V. radiata* hypocotyls when supplemented at the lowest concentrations (0.01 and 0.1 μM) while all the other compounds were ineffective (Table 2). In the adventitious rooting of apple cuttings all the compounds were ineffective (data not shown). Then, the adventitious rooting adjuvant activity of PBU, of compounds **4, 5**, **8** and **9** was assayed using the apple stem slice test, an endogenous growth regulators-free system performing a reproducible rooting response highly medium-dependent (Van der Krieken et al., 1993), either alone or in the presence of 1 μM IBA as exogenous auxin. When the compounds were supplemented alone, it was impossible to detect any biological activity, the slices browning as in hormone free condition. When supplemented in the simultaneous presence of IBA, PBU showed the same behaviour previously reported in *Arabidopsis* etiolated hypocotyls, enhancing at 4 μM then inhibiting at 16 μM the percentage of rooted slices. Compound **4** was completely ineffective. Compound **5** enhanced the percentage of rooting at all the concentrations tested. Compounds **8** and **9** enhanced the percentage of rooting at 4 and 16 μM (Table 2).

3. Conclusions

A few years ago we reported the cytokinin-like activity of *N*-phenyl-*N*′-benzothiazol-6-ylurea (PBU), a synthetic urea derivative structurally related to thidiazuron. Here we report for the first time that PBU is able to interact with the cytokinin receptor CRE1/AHK4 at high concentrations, allowing us to confirm PBU as a "weak" urea-type cytokinin, in comparison to TDZ that interacts at lower concentrations with the same receptor. It is clear that the cytokinin-like activity of PBU is dependent on selective interactions with its biological target as it is shown by the dramatic abolition of any cytokinin activity upon even minor structural modifications (see compounds **1–9**). On the other hand, our data suggest that further investigations are needed to elucidate the mode of action of PBU, as a possible interaction with AHK2 or/and AHK3 cytokinin receptors, or a hypothetical inhibition of cytokinin oxidase. Moreover, a higher affinity for the CRE1/AHK4 receptor could be obtained on the basis of structure-based modelling studies.

Among the PBU derivatives, only the symmetric ones were able to enhance adventitious rooting to a different extent in various bioassays, with a not fully understood mechanism. However, it has been once more confirmed that there is a close connection between the chemical structure of some urea derivatives and their behaviour as adventitious rooting adjuvants. It is noteworthy that the linkage NHCSNH that negatively affects the cytokinin-like activity, does not cancel the adventitious rooting adjuvant activity.

4. Experimental

4.1. General experimental procedures

Melting points (mp °C) were determined with a Buchi 512 apparatus and are uncorrected. Elemental analysis was performed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N, and S. The values found for C, H, N, S were within ±0.4% of the theoretical ones. ^{1}H NMR spectra of the newly synthesised compounds, in DMSO-d_6 solutions, were recorded on a Bruker AV 300 instrument at 298 K. Chemical shifts are reported as δ (ppm) relative to TMS as internal standard; coupling constants J are expressed in Hz. The progress of the reactions was monitored by thin layer chromatography with F_{254} silica-gel precoated sheets (Merck, Darmstadt, Germany). UV light was used for detection. For all target ureas: TLC eluent = CH_2Cl_2/MeOH 99:1.

4.2. Chemicals

Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting material, reagents and solvents were purchased from Aldrich Chemical Co. and from Fluka. IBA, TDZ and 6-benzylaminopurine (BAP) were purchased from Duchefa (The Netherlands).

4.3. Synthesis

4.3.1. General procedure for the synthesis of 4-, 5-, and 7-nitrobenzothiazoles

The appropriately substituted 2-chloronitroaniline (170 mmol) was refluxed in a mixture of formic acid – acetic anhydride 1:1 (23 ml) for 3 h. The mixture was cooled to room temperature and the solid residue was filtered off. $Na_2S{\cdot}9H_2O$ (246 mmnol) was added to the solid suspended in EtOH (40 ml) and the mixture was again refluxed for 3 h. The solvent was evaporated under reduced pressure and the residue was acidified with conc. HCl to obtain a yellow precipitate. The crude product was purified by flash chromatography (SiO_2, CH_2Cl_2).

4.3.2. Procedure for the synthesis of 6-nitrobenzothiazole

Benzothiazole (53 mmol) was added slowly, dropwise, to 30 ml of concentrated sulphuric acid, previously chilled to 0 °C, then 15 ml of concentrated nitric acid (333 mmol) were added dropwise maintaining the temperature below 0 °C. Finally the reaction was allowed to warm up to room temperature and stirred for 12 h. The reaction mixture was poured into ice water and crushed ice to produce a yellow precipitate, constituted of 6-nitroderivative as main product, that was filtered under vacuum and crystallized from EtOH (75%).

4.3.3. General synthesis of aminobenzothiazoles

A stirred mixture of the suitable nitrobenzothiazole (6 mmol), iron powder (21 mg-atom), HOAc (2.5 ml) and EtOH (20 ml) was heated under reflux for 3 h, and then poured in water and crushed ice. The water layer was extracted with CH_2Cl_2; the organic phase was washed with a solution of 10% NaOH, water and then dried over anhydrous sodium sulphate. Evaporation of the solvent under reduced pressure afforded a green solid.

*4.3.4. Synthesis of N-phenyl-N′-benzothiazolylureas (PBU, **1–3**)*

The target ureas were prepared according to the procedure described for PBU by Ricci et al. (2001a) starting from the appropriate aminobenzothiazole. Briefly, to a boiling solution of the amine (5.3 mmol) dissolved in anhydrous toluene (20 ml), phenylisocyanate (7.3 mmol) was added in one batch. The mixture was stirred under reflux for 1 h, then filtered and the collected white solid was recrystallised from the appropriate solvent.

*4.3.4.1. 1-(Benzo[d]thiazol-4-yl)-3-phenylurea(**2**).* Yield: 40%; mp 184–185 °C (EtOH). ^{1}H NMR (DMSO-d_6 δ, ppm): 9.57 (s, 1H, NH); 9.38 (s, 1H, NH); 9.22 (s, 1H, CH); 8.32 (d, 2H, J = 8.1, Ar); 7.70 (d, 1H, J = 7.5, Ar); 7.50 (d, 2H, J = 8.1, Ar); 7.42 (t, 2H, J = 8.1, Ar); 7.30 (d, 1H, J = 8.1, Ar) 6.99 (t, 1H, J = 7.2, Ar). Anal. Calcd. for $C_{14}H_{11}N_3SO$ (269.33): C, 62.43; H, 4.12; N, 15.60; S, 11.90. Found C, 62.17; H, 4.10; N, 15.52; S, 11.92%.

*4.3.4.2. 1-(Benzo[d]thiazol-7-yl)-3-phenylurea (**3**).* Yield: 55%; mp 248–250 °C (EtOH). ^{1}H NMR (DMSO-d_6 δ, ppm): 9.37 (s, 1H, CH); 8.99 (s, 1H, NH); 8.81 (s, 1H, NH); 7.88 (d, 1H, J = 8.4, Ar); 7.80 (d, 1H, J = 8, 1 Ar); 7.53–7.48 (m, 3H, Ar); 7.31 (d, 2H, J = 7.6, Ar); 7.00 (t, 1H, J = 7.2, Ar). Anal. Calcd. for $C_{14}H_{11}N_3SO$ (269.33): C, 62.43; H, 4.12; N, 15.60; S, 11.90. Found C, 62.20; H, 4.10; N, 15.61; S, 11.93%.

*4.3.5. Synthesis of N-N′-di(benzothiazolyl)ureas (**4, 5**)*

A solution of the appropriate aminobenzothiazole (6.6 mmol) and di-*t*-butyl dicarbonate (BOC_2O), (3.33 mmol) in 20 ml of anhydrous pyridine placed in a sealed vessel was heated at 115 °C for 18 h. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography (SiO_2, CH_2Cl_2/MeOH 95:5).

*4.3.5.1. 1,3-Di(benzo[d]thiazol-6-yl)urea (**4**).* Yield: 45%; mp 300–301 °C (DMF/H_2O). ^{1}H NMR (DMSO-d_6 δ, ppm):.9.22 (s, 2H, CH); 9.05 (s, 2H, NH); 8.38 (d, 2H, J = 1.5, Ar); 8.01 (d, 2H, J = 9.0, Ar); 7.52 (dd, 2H, J = 2.1 J = 8.7, Ar); 7.30 (d, 1H, J = 8.1, Ar). Anal. Calcd. for $C_{15}H_{10}N_4OS_2$ (326.40): C, 55.20.51; H, 3.09; N, 17.18; S, 19.65. Found C, 54.96; H, 3.11; N, 16.91; S, 19.41%.

*4.3.5.2. 1,3-Di(benzo[d]thiazol-5-yl)urea (**5**).* Yield: 40%; mp 306–308 °C (DMF/H_2O). ^{1}H NMR (DMSO-d_6 δ, ppm): 9.35 (s, 2H, CH); 8.99 (s, 2H, NH); 8.34 (s, 2H, Ar); 8.05 (d, 2H, J = 8.7, Ar); 7.51 (d, 2H, J = 9.0, Ar). Anal. Calcd. for $C_{15}H_{10}N_4OS_2$ (326.40): C, 55.20.51; H, 3.09; N, 17.17; S, 19.65. Found C, 60.43; H, 3.44; N, 12.15; S, 19.11%.

*4.3.6. Synthesis of thioureas (**6–9**)*

To a boiling solution of the appropriate amine (3.3 mmol) dissolved in anhydrous toluene (20 ml), phenylisothiocyanate (3.65 mmol) was added in one batch. The mixture was stirred under reflux for 6 h. The solvent was removed under reduced pressure and the crude product purified by flash chromatography (SiO_2, CH_2Cl_2/MeOH 99:1) afforded a first fraction (major) constituted of *N*-phenyl benzothiazolylthiourea (55%) and a second fraction (minor) constituted of *N-N′*-di(benzothiazolyl)thiourea.

*4.3.6.1. 1,3-Di(benzo[d]thiazol-6-yl)thiourea (**8**).* Yield: 38%; mp 202–203 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6 δ, ppm): 10.10 (s, 2H, NH); 9.33 (s, 1H, CH); 8.30 (d, 2H, J = 2.1, Ar); 8.03 (d, 2H, J = 9.0, Ar); 7.58 (dd, 2H, J = 2.4 J = 8.7, Ar); Anal. Calcd. for $C_{15}H_{10}N_4S_3$ (342.69): C, 52.61; H, 2.94; N, 16.36; S, 28.09. Found C, 52.61; H, 3.38; N, 15.36; S, 27.95%.

*4.3.6.2. 1,3-Di(benzo[d]thiazol-5-yl)thiourea (**9**).* Yield: 35%; white crystals, mp 191–193 °C (EtOH/H_2O). ^{1}H NMR (DMSO-d_6 δ, ppm): ^{1}H NMR (DMSO-d_6 δ, ppm): 10.13 (s, 2H, NH); 9.40 (s, 2H, CH); 8.24 (d, 2H, J = 1.5 Ar); 8.30 (d, 2H, J = 2.1 Ar); 8.11 (d, 2H, J = 8.4 Ar); 7.57 (dd, 2H, J = 2.4 J = 8.7 Ar). Anal. Calcd. for $C_{15}H_{10}N_4S_3$ (342.69): C, 52.61; H, 2.94; N, 16.36; S, 28.09. Found C, 52.61; H, 3.38; N, 15.36; S, 27.95%.

4.4. Cytokinin and adventitious rooting bioassays

All the tested compounds were dissolved in dimethylsulphoxide (DMSO) and the final concentration of DMSO in the culture medium or in the aqueous solutions did not exceed 0.2% (Schmitz and Skoog, 1970).

4.4.1. Tomato explant regeneration assay

Twelve cotyledon explants, obtained from *in vitro* cultured seedlings, were plated on MS medium (Murashige and Skoog, 1962) containing the PBU derivatives at 1, 5 and 10 μM, both alone and in the presence of 20 μM 1,2-benzisoxazole-3-acetic acid (BOAA), as auxin (Ricci et al., 1996). Control was performed by cotyledon explants cultured in the presence of TDZ, as cytokinin-like urea derivative, in the same culture conditions. After 2 week incubation at light intensity of 27 μmol m^{-2} s^{-1} at 26 °C under 16 h photoperiod, the cotyledon explants were transferred to a hormone free medium and the number of explants that regenerated shoots was checked 2 weeks later. The experiments were carried out in triplicate, and repeated three times.

4.4.2. Amaranthus assay

The *Amaranthus caudatus* L. bioassay was performed as previously described (Ricci et al., 2006). Four different concentrations 10 nM, 100 nM, 1 μM and 10 μM of the test compounds were assayed. Control was performed with phosphate buffer alone (hormone free, HF). For each experiment the same concentrations of 6-benzylaminopurine (BAP) were also tested and the experiment was considered "valid" only in the presence of a classical BAP concentration-course variation. The experiments were carried out in triplicate and repeated three times. The results were expressed as a percentage of the control (phosphate buffer alone) by the formula (tested-control/control) × 100 [(T − C/C) × 100].

4.4.3. Heterologous bacterial assay

E. coli strain KMI001, harbouring vector pINIII-AHK4 expressing the *Arabidopsis* cytokinin receptor CRE1/AHK4, that is capable of signalling the downstream YojN → RcsB → *cps::lacZ* pathway in response to external cytokinins, was used in the experiments (Suzuki et al., 2001; Yamada et al., 2001). Bacterial strains were kindly provided by dr. T. Mizuno (Nagoya, Japan). Bacteria were

grown in Luria broth at 37 °C with ampicillin (50 μg/ml) with extensive shaking. Culture density was controlled by measuring the absorption at 600 nm (OD_{600}). A homogenous bacterial suspension (OD_{600} between 0.04 and 0.4) was aliquoted in multiwell dishes supplemented with 1, 10, and 100 μM of the test compounds. Bacteria grown in the presence of the same concentrations of TDZ and of the relative volumes of DMSO were used as control. After 40 h incubation at 25 °C in the dark, the β-galactosidase activity was detected as described by Zhang and Bremer (1995) with minor modifications. Twenty μl samples of cultures were mixed into 80 μl of permeabilization solution (0.8 mg/ml hexadecyltrimethylammonium bromide, 0.4 mg/ml sodium deoxycholate, 100 mM Na_2HPO_4, 20 mM KCL, 2 mM $MgSO_4$, 5.4 μl/ml β-mercaptoethanol). This mixture was kept at 30 °C for 1.30 h, then 600 μl of 30 °C prewarmed substrate solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 2.7 μl/ml β-mercaptoethanol) were added to initiate the reaction. After 2 h at 30 °C, reactions were stopped by the addition of 700 μl of 1 M Na_2CO_3 and the absorption at 420 nm was recorded for each sample. Enzyme activities were expressed as Miller units (Miller, 1972) by the formula $1000 \times [OD_{420}/(OD_{600} \times 20\ \mu l \times 120\ min)]$.

4.4.4. Adventitious rooting of Arabidopsis seedlings

A. thaliana ecotype Columbia (Col-0) seedlings were grown as previously described (Sorin et al., 2005) with minor modifications. Seeds were surface sterilized in 70% ethanol for 1 min, followed by 10 min in 50% commercial bleach (equivalent to 2.5% NaOCl), washed five times in sterile distilled water, sown on 1/4 strength Murashige and Skoog (MS) medium supplemented with 0.8% agar, pH 5.8 (germination medium) and kept in the dark at 26 °C. Groups of 10 three-day-old etiolated seedlings were transferred to Petri dishes containing full strength MS medium, supplemented with MS vitamins, 3% sucrose, 0.8% agar, pH 5.8, under different rooting conditions. The medium was supplemented with 1, 2, 4 or 8 μM of the test compounds alone and in the simultaneous presence of 0.1 μM IBA, as auxin. Medium containing DMSO or 0.1 μM IBA were used as controls. Petri dishes were placed vertically in a growth chamber at light intensity of 27 $\mu mol\ m^{-2}\ s^{-1}$ at 26 °C under 16 h photoperiod. Emergent adventitious roots on the hypocotyls were scored at 7 days after transfer to the light using a stereomicroscope. The experiments were done in triplicate and repeated twice. The results were expressed as a percentage of the control (hormone free or 0.1 μM IBA, respectively) by the formula (tested-control/control) × 100 [(T − C/C) × 100].

4.4.5. Adventitious rooting of mung bean shoots

Seeds of mung bean (*V. radiata* L.) were grown as previously described (Ricci et al., 2006). The shoots were harvested by cutting 3 cm under the cotyledonary node and deprived of cotyledons when present. Then they were cultured in darkened shell vials containing 15 ml of aqueous solution of the test compounds separately at 0.01, 0.05, 0.1, 0.5, 1 and 5 μM, and incubated at light intensity of 27 $\mu mol\ m^{-2}\ s^{-1}$ at 26 °C under 16 h photoperiod for the initial 24 h. The shoots were then transferred to distilled water and incubated under the same culture conditions described above. Control was performed with mung bean shoots cultured in distilled water (HF) at light intensity of 27 $\mu mol\ m^{-2}\ s^{-1}$ at 26 °C under 16 h photoperiod. All the experiments were carried out using 10 shoots, repeated three times and the number of roots was counted after 1 week. The results were expressed as a percentage of the control (H_2O alone) by the formula (tested-control/control) × 100 [(T − C/C) × 100].

4.4.6. Adventitious rooting of apple microcuttings

In vitro shoot cultures of *Malus pumila* Mill. rootstock M26 were maintained as previously described (Ricci et al., 2006). Individual shoots, 2–2.5 cm in length, were used either for further propagation or for microcutting rooting experiments or for preparing 1-mm thick stem slices. Malus M26 microcuttings, 2–2.5 cm in length excised from 4-week old cultures, were incubated in MP medium in the presence of 1, 4, 8, 16 μM of the test compounds without any exogenous auxin supplementation, and then cultured at light intensity of 27 $\mu mol\ m^{-2}\ s^{-1}$ at 26 °C under 16 h photoperiod. Control was performed with microcuttings cultured in hormone free (HF) medium under the same culture conditions. In all the experiments the roots were counted after 4 weeks. For each experiments 50 microcuttings were used. The results are shown as number of roots per rooted microcuttings (total induced roots divided by the number of rooted microcuttings).

4.4.7. Adventitious rooting of apple stem slices

Malus M26 stem slices were prepared and distributed over different rooting treatments as previously reported (Ricci et al., 2006). Groups of 16 slices were cultured in Petri dishes with the apical side on a nylon mesh put on the above described culture medium supplemented with 1, 2, 4 or 8 μM of the test compounds alone or in the simultaneous presence of 1 μM IBA, as auxin. The dishes were incubated upside down in the darkness for 6 days. Simply removing the nylon mesh, all the slices were then transferred to fresh culture medium lacking of the test compounds and IBA; the dishes were incubated at 26 °C at light intensity of 27 $\mu mol\ m^{-2}\ s^{-1}$ under 16 h photoperiod. Control was performed with stem slices cultured in the presence of 1 μM IBA alone and slices were subjected to the same manipulations described above. The number of rooted slices in relation to total slices in specific treatments and the induced number of roots were counted after 14 days. The percentage of rooting was expressed as a percentage of the control (1 μM IBA) by the formula (tested-control/control) × 100 [(T − C/C) × 100]. All the experiments were carried out in triplicate and repeated twice.

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