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(71) Applicants: **UNIVERZITA PALACKEHO V OLOMOUCI** [CZ/CZ]; Krizkovskeho 8, 771 47 Olomouc (CZ). **SYNGENTA PARTICIPATIONS AG** [—/CH]; Schwarzwaldallee 215, CH-4058 Basel (CH).

(72) Inventors: **ZATLOUKAL, Marek**; Revolucni 18a, 78701 Sumperk (CZ). **PLIHALOVA, Lucie**; Handkeho 1a/757, 779 00 Olomouc (CZ). **KLASKOVA, Jana**; Hodolanska 24a, 77200 Olomouc (CZ). **SPICHAL, Lukas**; Tr. Svornosti 14, 77900 Olomouc (CZ). **KOPRNA, Radoslav**; Sokolovska 161/2B, 77900 Olomouc (CZ). **DOLEZAL, Karel**; Postlucho 31, 78365 Hlubocky (CZ). **STRNAD, Miroslav**; Zapadni 25, 77900 Olomouc (CZ).

(74) Agent: **HARTVICOVA, Katerina**; INVENTIA s.r.o., Na Belidle 3, 15000 Praha 5 (CZ).

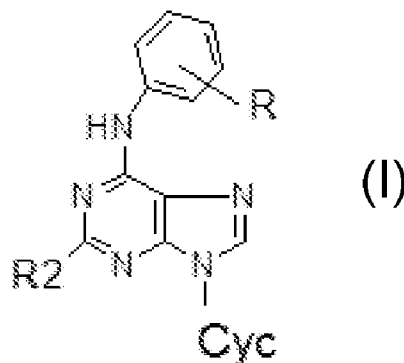
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(54) Title: SUBSTITUTED 6-ANILINO-9-HETEROCYCLYLPUURINE DERIVATIVES FOR INHIBITION OF PLANT STRESS



(57) Abstract: The invention relates to substituted 6-anilino-9-heterocyclylpurine derivatives of formula (I) wherein R denotes one to five substituents independently selected from the group comprising hydrogen, halogen, hydroxy, amino, alkyloxy and alkyl group, R2 is selected from the group comprising amino, halogen, hydroxy, thio, and alkylthio group, Cyc is five- or six-membered heterocyclic ring containing one oxygen atom. Said derivatives are useful for inhibition of plant stress.

Substituted 6-anilino-9-heterocyclylpyrine derivatives for inhibition of plant stressTechnical Field

- 5 The invention relates to 2-substituted-6-anilino-9-heterocyclylpyrine derivatives, their use in agriculture and preparations containing these derivatives.

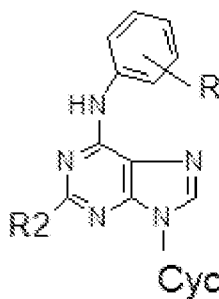
Background Art

- 10 Cytokinins are plant hormones that regulate a large number of developmental and physiological processes in plants. With the exception of diphenylurea, all naturally occurring cytokinins are 6-substituted purine derivatives that can be further modified by substitution. Cytokinin oxidase/dehydrogenase (CKX) is an enzyme that is responsible for cytokinin degradation. The modulation of cytokinin levels by either exogenous application of cytokinins or regulation of their
- 15 endogenous levels genetically through cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) have already shown possible applications in agriculture. CKX inhibitors are cytokinin derivatives that can delay cytokinin degradation and therefore prolong the period of cytokinin action. For example, exogenous application of cytokinins led to shortening of the time to anthesis in tomato (Sawhney and Shukla, Am J Bot 81:1640, 1994) or reversion of male sterility in barley (Ahokas, Proc. Natl.
- 20 Acad. Sci. USA 79:7605, 1992). Anther- and pollen-specific expression of CKX in maize was shown to be a potential tool for generating male sterility for production of hybrid varieties of traditionally non-hybrid crops. Recent work reported CKX involvement in regulation of rice grain production (Ashikari et al., Science 309:741, 2005).

- We have recently discovered that novel generations of CKX inhibitors could be based on 2-
- 25 substituted 6-anilinopurines (CZ302225B6). It is an object of the present invention to provide further compounds having improved selectivity and efficiency index in plants, i.e., having lower toxicity and higher activity than known active substances. The compounds of this invention exhibit surprisingly strong antistress and antisenescence properties on plants.

30 Disclosure of the Invention

The object of this invention are 2-substituted-6-anilino-9-heterocyclylpyrines of the general formula I



(I)

and pharmaceutically acceptable salts thereof with alkali metals, ammonium or amines, in the form of racemates or optically active isomers, as well as their addition salts with acids, wherein

R denotes one to five substituents independently selected from the group comprising hydrogen, halogen, hydroxy, amino, alkyloxy and alkyl group,

R2 is selected from the group comprising amino, halogen, hydroxy, thio, and alkylthio group,

Cyc is five- or six-membered heterocyclic ring containing one oxygen atom.

Cyc is preferably selected from tetrahydropyranyl and tetrahydrofuranyl, more preferably from tetrahydropyran-2-yl and tetrahydrofuran-2-yl.

R2 is preferably selected from the group comprising chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio.

5 The generic substituent groups have meanings as follows:

amino denotes the group $-NH_2$,

hydroxy denotes the group $-OH$,

halogen denotes an atom selected from the group comprising fluorine, bromine, chlorine and iodine atom,

10 thio denotes the group $-SH$,

alkyl denotes branched or unbranched alkyl group containing 1 to 6 carbon atoms, preferably 1 to 4 carbon atoms,

hydroxy denotes the group $-OH$,

alkyloxy denotes the group $-OR_a$, wherein R_a is alkyl,

15 alkylthio denotes the group $-SR_b$, wherein R_b is alkyl.

In accordance with the invention, substituted 6-anilino-9-heterocyclyl-purine derivatives of the general formula **I** are selected from: 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-anilino-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro,

[illegible]

[illegible]

(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-hydroxy-4-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxy-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxy-5-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(4-hydroxy-3,5-dimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloro-4-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloro-5-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloro-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-bromo-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-methoxy-3-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-methoxy-4-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, and salts thereof with alkali metals, ammonium or amines, in the forms of racemates or optically active isomers, as well as their addition salts with acids.

The following substituted 6-anilino-9-heterocyclylpyrune derivatives are particularly preferred, namely: 2-(chloro, fluoro, bromo)-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-hydroxy-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-chloro-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-bromo-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(4-hydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, and salts thereof with alkali metals, ammonium or amines, in the forms of racemates or optically active isomers, as well as their addition salts with acids.

The present invention further includes antisenescence and/or antistress preparations comprising at least one compound of general formula I or salts thereof with alkali metals, ammonium or amines, in the forms of racemates or optically active isomers, as well as their addition salts with acids, and auxiliary substances.

The invention further includes antisenescent and/or antistress preparations for plants, plant organs and plant cells, comprising at least one compound of the general formula **I** or a salt thereof, and at least one auxiliary substance.

- 5 A further aspect of this invention is use of at least one substituted 6-anilino-9-heterocyclpurine derivative of general formula **I** for inhibiting stress in whole plants, plant organs, plant tissues and/or plant cells.

A further aspect of this invention is use of at least one substituted 6-anilino-9-heterocyclpurine derivative of general formula **I** for stimulation of growth and/or development of plant shoots
10 without inhibiting growth and/or development of roots.

A further aspect of this invention is use of at least one substituted 6-anilino-9-heterocyclpurine derivative of general formula **I** as plant antistress factor, in particular for increasing the yield and quality of agricultural product in harmful conditions. Harmful conditions may include drought,
15 heat, salinity, light, freezing and flowage stress. The use as plant antistress factor relates in particular to agricultural use.

A further aspect of the invention is a method of inhibiting and/or preventing senescence and/or stress in plants, plant organs, plant tissues and/or plant cells, comprising the application of at least
20 one 2-substituted-6-anilino-9-heterocyclpurine derivative of the general formula **I** or a salt thereof for to said whole plant, plant organs, tissues and cells.

Another aspect of the invention is a method of stimulation of plant shoot growth and/or development without inhibition of root growth and/or development, comprising the application of
25 at least one 2-substituted-6-anilino-9-heterocyclpurine derivative of the general formula **I** or a salt thereof to a plant, a seedling or a seed.

A further aspect of the invention is use of 2-substituted-6-anilino-9-heterocyclpurine derivatives of the general formula **I** in tissue cultures for stimulation of proliferation and morphogenesis.
30

A further aspect of the invention is use of 2-substituted-6-anilino-9-heterocyclpurine derivatives of the general formula **I** as plant antistress factors in agriculture, in particular to increase crop production in stress conditions to dispatch grain filling and to increase grain and fruit size of plants, to shorten plant seed germination period, to increase the yield and quality of agricultural products,
35 to enhance root and shoot growth, and to increase stress resistance to environmental factors. The

compounds of this invention show stimulation of plant shoot growth and development without root growth inhibition.

A further particular aspect of the invention is the use of 2-substituted-6-anilino-9-heterocyclylpyrine derivatives of the general formula **I** as plant antistress factors for decreasing of stress-induced senescence in the production of crops, in particular cereals (wheat, barley, rice, maize, rye, oat, sorghum, and related species), beet (sugar beet and foddered beet); pomes, drupes and soft fruits (apples, pears, plums, peaches, almonds, cherries, strawberries, raspberries and blackberries); leguminous plants (beans, lentils, peas, soybeans); oil plants (rape, mustard, poppy, olives, sunflowers, coconut, *Ricinus*, cocoa beans, groundnuts); cucumber plants (pumpkins, cucumbers, melons); fibre plants (cotton, flax, hemp, jute); citrus fruit (oranges, lemons, grapefruit, mandarins); vegetables (spinach, cinnamomum, camphor) or plants such as tobacco, nuts, eggplants, sugar cane, tea, vine grapes, hops, bananas and natural rubber and medicinal plants, as well as ornamentals. Crops include those which have been rendered tolerant towards classes of growth factors by conventional breeding methods or genetic engineering methods.

A further aspect of the invention is the use of 2-substituted-6-anilino-9-heterocyclylpyrine derivatives of the general formula **I** as plant antistress factors for delaying the aging and senescence of plant cells, plant tissues, plant organs and/or the whole plants.

20

A further aspect of the invention is the use of 2-substituted-6-anilino-9-heterocyclylpyrine derivatives of the general formula **I**, for plant embryonic cells production and plant cloning.

The compounds of the general formula **I** are used in unmodified form or, preferably, together with the auxiliary substances conventionally employed in the art of preparations. To this end they are conveniently formulated as concentrates of active compounds as well as suspensions and dispersions, preferably isotonic water solutions, suspensions and dispersion, diluted emulsions, soluble powders, dusts, granulates, creams, gels, oil suspensions and also encapsulations, e.g. in polymeric substances. As with the type of the preparation, the methods of application, such as spraying, atomizing, dusting, scattering, coating or pouring, are chosen in accordance with the intended objectives and the prevailing circumstances. The preparations may be sterilized and/or contain further auxiliary substances of neutral character, such as preservatives, stabilizers, wetting agents, emulgators, solubilizing agents, fertilizers, micronutrient donors or other substances for obtaining special effects.

35

The compounds of the formula I can be mixed with other plant growth regulators, herbicides, fungicides and insecticides, resulting in synergistic activities.

PREPARATIONS

The preparations comprising the compounds (active ingredients) of formula I, optionally in the form of salts, and, where appropriate, one or more solid or liquid auxiliary substances, are prepared in a manner known *per se*, e.g. by intimately mixing and/or grinding the active ingredients with the auxiliary substances, e.g. solvents or solid carriers. In addition, surface-active compounds (surfactants) may also be used in the preparation of the formulations.

Depending on the nature of the compound of formula I to be formulated, suitable surface-active compounds are non-ionic, cationic and/or anionic surfactants and surfactant mixtures having good emulsifying, dispersing and wetting properties. Examples of suitable anionic, non-ionic and cationic surfactants are listed, for example, in WO 97/34485.

Also suitable in the preparation of the growth regulator compositions according to the invention are the surfactants conventionally used in formulation technology, which are described, *inter alia*, in "McCutcheon's Detergents and Emulsifiers Annual" MC Publishing Corp., Ridgewood New Jersey, 1981, Stache, H., "Tensid- Taschenbuch", Carl Hanser Verlag, MunichNienna, 1981 and M. and J. Ash, "Encyclopedia of Surfactants", Voll-III, Chemical Publishing Co., New York, 1980-81. The preparations usually contain from 0.1 to 99 % by weight, especially from 0.1 to 95 % by weight, of active ingredient mixture comprising a compound of formula I, from 1 to 99.9% by weight of a solid or liquid adjuvant, and from 0.1 to 25 % by weight, especially from 0.1 to 25 % by weight, of a surfactant.

Whereas commercial products are usually formulated as concentrates, the end user will normally employ dilute formulations. The composition can thus contain also further additives, such as stabilizers, e.g., plant oils or epoxidized plant oils (epoxidized palm oil 0;1, rapeseed or olive oil), defoamers, e.g., silicon oil, preservatives, wetting agents or emulgators, viscosity agent, binders, glues, and also fertilizers and other active additives. Preferred formulations have especially the following compositions: (% = percent by weight)

30 Emulsifiable concentrates:

active ingredient mixture:	1 to 90 %, preferably 5 to 20 %
surfactant:	1 to 30 %, preferably 10 to 20 %
liquid carrier:	5 to 98 %, preferably 60 to 85 %

Dusts:

35 active ingredient mixture:	0.1 to 10 %, preferably 0.1 to 5 %
solid carrier:	99.9 to 90 %, preferably 99.9 to 99 %

Suspension concentrates:

active ingredient mixture:	5 to 75 %, preferably 10 to 50 %
water:	94 to 24 %, preferably 88 to 30 %
surfactant:	1 to 40 %, preferably 2 to 30 %

5 Wettable powders:

active ingredient mixture:	0.5 to 90 %, preferably 1 to 80 %
surfactant:	0.5 to 20 %, preferably 1 to 15 %
solid carrier:	5 to 99 %, preferably 15 to 90 %

Granules:

10 active ingredient mixture:	0.1 to 30 %, preferably 0.1 to 15 %
solid carrier:	99.9 to 70 %, preferably 99.9 to 85 %

The compositions may also comprise further ingredients, such as stabilisers, e.g. vegetable oils or epoxidised vegetable oils (epoxidised coconut oil, rapeseed oil or soybean oil), anti-foams, e.g. 15 silicone oil, preservatives, viscosity regulators, binders, tackifiers, and also fertilisers or other active ingredients. For the use of growth factors of formula I, or of compositions comprising them, various methods and techniques come into consideration, such as, for example, the following:

i) Seed dressing

20 a) Dressing of the seeds with a wettable powder formulation of a compound of the general formula **I** by shaking in a vessel until uniformly distributed over the seed surface (dry dressing). In that procedure approximately from 1 to 500 g of compound of the general formula **I** (4 g to 2 kg of wettable powder) are used per 100 kg of seed.

25 b) Dressing of the seeds with an emulsifiable concentrate of a compound of formula **I** according to method a) (wet dressing).

c) Dressing by immersing the seeds for from 1 to 72 hours in a liquor comprising from 100 to 1000 ppm of a compound of formula **I** and optionally subsequently drying the seeds (immersion 30 dressing).

Dressing the seed or treating the germinated seedling are naturally the preferred methods of application, because treatment with the active ingredients is directed entirely at the target crop. Generally from 1 to 1000 g of antidote, preferably from 5 to 250 g of antidote, are used per 100 kg 35 of seed, but depending on the methodology, which also enables the addition of other active

ingredients or micronutrients; the concentration limits indicated can be varied up or down (repeat dressing).

ii) Application as a tank mixture

- 5 A liquid formulation of a mixture of antidote and growth regulator is used (ratio by weight of the one to the other from 10:1 to 1 :100), the rate of application of growth regulator being from 0.005 to 5.0 kg per hectare. Such tank mixtures are applied before or after sowing.

10 iii) Application to the seed furrow

The compounds of formula I are introduced into the open, sown seed furrow in the form of an emulsifiable concentrate, wettable powder or granules. Once the seed furrow has been covered over, the growth regulator is applied in the usual manner in the pre-emergence process.

15 iv) Controlled release of the active ingredient

The compounds of formula I are applied in solution to mineral granule carriers or polymerised granules (urea/formaldehyde) and dried. If desired, it is also possible to apply a coating that allows the active ingredient to be released in metered amounts over a specific period of time (coated granules).

20

Brief description of drawings

Figure 1 shows differences in dose-dependent activation of cytokinin receptors AHK3 (upper chart) and CRE1/AHK4 (lower chart) in *Escherichia coli* receptor assay (Spíchal et al., 2004) by
25 cytokinin trans-zeatin (tZ), inhibitors of cytokinin oxidase-dehydrogenase INCYDE (2-chloro-6-(3-methoxyanilino)purine) and INCYDE-F (2-fluoro-6-(3-methoxyanilino)purine) and representatives of the novel derivatives, compounds **12** and **91**. Values represent means of three repetitions, error bars represent SD.

Figure 2 shows differences in dose-dependent activation of cytokinin responsive reporter gene
30 ARR5:GUS (Romanov et al., 2002) by cytokinin 6-benzylaminopurine (BAP), inhibitors of cytokinin oxidase-dehydrogenase INCYDE and INCYDE-F and representatives of the novel derivatives compound **12** and **91**. Bars represent means of three repetitions, error bars represent SD.

Figure 3 shows comparison of the effect of compound **12** and CKX inhibitor INCYDE application
35 on complementation of wild-type phenotype of AtCKX1 Arabidopsis seedlings. (A) AtCKX1 control, (B) AtCKX1 cultivated on 0.1 μ M compound 12, (C) AtCKX1 cultivated on 0.1 μ M INCYDE, (D) control plant with wild-type phenotype.

Figure 4 illustrates similar dose-dependent effect of the compound **12** and structurally related CKX inhibitor INCYDE on complementation of wild-type shoot phenotype of AtCKX1 plants. Bars represent means of fresh weight of rosettes of treated plants, error bars indicate SD (n=30).

Figure 5 illustrates clear difference in effect of the compound **12** and structurally related CKX inhibitor INCYDE on stimulation of root biomass of AtCKX1 plants. Bars represent means of fresh weight of roots of treated plants, error bars indicate SD (n=30).

Figure 6 shows comparison of the effect of 0.1 μ M CKX inhibitor INCYDE (B), 0.1 μ M compound **12** (C) and cytokinin BAP (D) on wild-type Arabidopsis root growth and development (A) *in vitro*.

Figure 7 shows difference in dose-dependent effect of CKX inhibitor INCYDE, compound **12** and cytokinin BAP on retention of chlorophyll in rosettes incubated for 6 days in the dark in the presence of the tested compounds. Values represent means from six replicates, error bars represent SD.

Figure 8 illustrates the effect of compound **12** applied by drenching on shoot growth and biomass development of Arabidopsis plants.

Figure 9 shows the effect of compound **12** applied by drenching in different application rates on shoot growth and biomass development of Arabidopsis plants.

Figure 10 shows effect of seed coating with compound **12** on formation of root and shoot dry biomass of maize in pot experiment. Values are shown relative to control.

Figure 11 illustrates the effect of compound **12** on viability of human MRC-5 and BJ cell lines in Calcein AM assay after 3 days. Values are shown relative to control.

Figure 12 shows activity of compound **12** in Ames test (Ames MPFTM 98/100 Microplate Format Mutagenicity Assay); +/- S9 means with or without metabolic activation, final concentration of positive controls (labeled as +): for -S9: 2 μ g/ml 2-nitrofluorene + 0,1 μ g/ml 4-nitroquinoline-N-oxide, for +S9: 5 μ g/ml 2-aminoanthracene.

Figure 13 shows immunofluorescent detection of γ -H2AX (indicator of DNA damage) after the application of compound **12** for 24h, cell line U-2-OS, positive control: 0,5 - 2 Gy roentgen radiation (Baltobloc), * significantly different from control, t-test, $\alpha=0,01$.

30 Examples of Carrying out the Invention

The starting material for the compounds of the formula **I** is 2,6-dichloro-9-(tetrahydropyran-2-yl)purine or 2,6-dichloro-9-(tetrahydrofuran-2yl)purine prepared from 2,6-dichloropurine and 3,4-dihydro-2H-pyran or 2,3-dihydrofuran in acidic conditions of trifluoroacetic acid. Another starting material can be 2-amino-6-chloro-9-(tetrahydropyran-2-yl) or 9-(tetrahydrofuran-2-yl)purine or 2-fluoro-6-chloro-9-(tetrahydropyran-2-yl) or 9-(tetrahydrofuran-2-yl)purine, synthesised from 2-

amino-6-chloropurine by reaction with tetrafluoroboric acid in the presence of sodium nitrate aqueous solution (Beach et al., 1992). Another starting compound, 2-bromo-6-chloro-9-(tetrahydropyran-2-yl) and 9-(tetrahydrofuran-2-yl)purine, can be prepared from 2-amino-6-chloropurine (Kim Hak Sung et al.; J.Med.Chem.46; 23; 2003; 4974-4987). Yet another starting material can be 2-methyl-6-chloro-9-(tetrahydropyran-2-yl) or 9-(tetrahydrofuran-2-yl)purine (Kim et al., 2003). Yet another starting material can be 2-nitro-6-chloro-9-Boc-purine, which can be prepared from 6-chloropurine (Rodenko et al.; J .Am. Chem .Soc. 2005, 127, 5957-5963).

Starting substituted phenylamines, not commercially available (others obtained *via* Sigma Aldrich or Fluorochem), were prepared from the corresponding aldehydes in the presence of suitable catalyst. These, which have more hydroxyl groups, may also be prepared by demethylation of appropriate methoxyderivatives using 48% HBr in N₂ atmosphere.

Elemental analyses (C, H and N) were performed on an EA1108 CHN analyser (Fissons Instruments). The melting points were determined on a BÜCHI Melting Point B-540 apparatus. Analytical thin layer chromatography (TLC) was carried out using silica gel 60 WF₂₅₄ plates (Merck), mobile phase CHCl₃:MeOH: (9:1, v/v). ES+ mass spectra were recorded using direct probe on Waters ZMD 2000 mass spectrometer. The mass monitoring interval was 10 - 1500 amu. The spectra were collected using 3.0 second cyclical scans and applying sample cone voltage 25 V at source block temperature 150°C, desolvation temperature 80°C and desolvation gas flow rate 200 l/hour. The mass spectrometer was directly coupled to a MassLynx data system. NMR spectra were measured in a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz (¹H) and 75.48 MHz (¹³C), respectively. Samples were prepared by dissolving the compounds in DMSO-d₆. Tetramethylsilane (TMS) was used as the internal standard.

Example 1

2-Chloro-6-anilino-9-(tetrahydropyran-2-yl)purine

Aniline (4.96 g; 0.05 mol) was added to a suspension of 2,6-dichloro-9-(tetrahydropyran-2-yl)purine (8.19 g; 0.03 mol) in n-propanol (60 ml) and N,N'-ethyldiisopropylamine (6.44 g; 0.05 mol) was added. The reaction mixture was stirred at 100 °C for 5 hours. After cooling to room temperature the precipitate was filtered off, washed with n-propanol (2x10 ml) and water (3x10 ml) and dried in the drying oven at 60 °C into constant weight. Yield: 6.26 g yellowish substance (84.9 %). TLC (chloroform-methanol; 9:1): one single spot; free of the starting material, HPLC purity: 98+ %. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.98d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 4.02d (1H, THP), 5.63d (1H, THP), 7.09t, J = 8.0Hz (1H, Ar-H), 7.37t, J = 8.0Hz (2H, Ar-H), 7.84d, J = 8.0Hz (2H, Ar-H), 8.30s (1H, C8-H), 10.18s (1H, N6-H).

Example 2

2-Chloro-6-(3-chloroanilino)-9-(tetrahydropyran-2-yl) or (tetrahydrofuran-2-yl)purine

2,6-dichloro-9-(tetrahydropyran-2-yl)purine (8,19 g; 0.03 mol) reacted with 3-chloroaniline (3.83 g; 0.03 mol) in n-butanol (40 ml) in the presence of triethylamine (7 ml; 0.05 mol) at 90°C for 5 hours. After cooling to room temperature, the yellow precipitate was filtered off, washed with cold n-butanol (2x10 ml), water (3x10 ml) and dried in the drying oven at 60 °C into constant weight. Yield: 5.69 g yellow powder (65.8 %). The crude product was crystallized from isopropanol. TLC (chloroform-methanol; 9:1): one single spot; free of starting material. HPLC purity: 98+ %.

2-chloro-6-(3-chloroanilino)-9-(tetrahydrofuran-2-yl)purin was prepared in an analogical way from 2,6-dichloro-9-(tetrahydrofuran-2-yl)purine and 3-chloroaniline. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.98d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 4.02d (1H, THP), 5.63d (1H, THP), 7.11dd, J_a = 8.2Hz, J_b = 2.4Hz (1H, Ar-H), 7.37t, J=8.2Hz, (1H, Ar-H), 7.82dd, J_a = 8.2Hz, J_b = 2.1Hz (1H, Ar-H), 8.06t, J=2.1Hz (1H, Ar-H), 8.34s (1H, C8-H), 10.37bs (1H, N6-H).

Example 3

2-Chloro-6-(3-fluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

This compound was prepared in a similar manner as described in example 2, by reaction of 2,6-dichloro-9-(tetrahydropyran-2-yl) or (9-tetrahydrofuran-2yl)purine (8,19 g; 7,77 g 0.03 mol) with 3-fluoroaniline (3.33 g, 0.03 mol). The reaction mixture was then evaporated on a rotary vacuum evaporator and the residue was partitioned between ethyl acetate and 0.5 M HCl. The organic layer was washed with water, dried with MgSO₄, and evaporated to give a yellow solid (3.72 g). The crude product was purified by flash chromatography. Yield: 2.20 g yellow crystalline powder. TLC (chloroform-methanol; 9:1): homogenous. HPLC purity: 98+ %. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.98d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 4.02d (1H, THP), 5.63d (1H, THP), 6.59dd, J_a = 8.0Hz, J_b = 2.5Hz (1H, Ar-H), 7.02d, J = 2.5Hz (1H, Ar-H), 7.25dd, J_a = 8.0Hz, J_b = 2.5Hz (1H, C15-H), 7.32tt, J_a = 8.0Hz, J_b = 2.5Hz (1H, Ar-H), 8.28s (1H, C8-H), 11.17s (1H, N6-H).

Example 4

2-Chloro-6-(3-hydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

The compound was prepared by the reaction of 2,6-dichloro-9-(tetrahydropyran-2-yl) or (9-tetrahydrofuran-2yl)purine (8,19 g; 7,77 g 0.03 mol), 3-aminophenol (3.27 g; 0.03 mol), and N,N-ethyldiisopropylamine (6.44 g; 0.05 mol) in n-butanol (40 ml) at 90 °C for 4 hours. The reaction mixture was then allowed to cool to room temperature and stirred. The formed crystalline precipitate was collected, rinsed with cold n-butanol (3x10 ml), water (3x10 ml) and dried in the

oven into constant weight. TLC (chloroform-methanol; 9:1): homogenous. HPLC purity: 98+ %. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.97d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 4.02d (1H, THP), 5.63d (1H, THP), 7.13t (1H, Ar-H), 7.28d (1H, Ar-H), 7.38s (1H, Ar-H), 8.30s (1H, C8-H), 9.44s (1H, N6-H), 10.09s (1H, OH).

5

Example 5

2-Chloro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

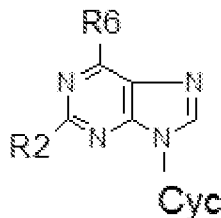
To a suspension of 2,6-dichloro-9-(tetrahydropyran-2-yl) or 9-(tetrahydrofuran-2-yl)purine (8.19 g; 7.77 g 0.03 mol) and m-anisidine (3.69 g; 0.03 mol) in n-pentanol (40 ml), triethylamine (7 ml; 0.05 mol) was added. The reaction mixture was stirred at 90°C for 4 hours and then it was allowed to cool to the room temperature. The white precipitate was collected, washed with isopropanol (2x10 ml) and water (3x10 ml). The crude product was purified by the crystallization from methanol in the presence of activated charcoal to give white crystals. Yield: 4.36 g (77.8 %). TLC (chloroform-methanol; 9:1): one single spot, free of starting material, HPLC purity: 98+ %

Alternative procedure:

To a suspension of 2-chloro-6-(3-methoxyanilino)purine (10 g; 0.036 mol) in dioxane (250 ml) and 3,4-dihydropyran (10 ml; 0.1 mol), trifluoroacetic acid (10 ml; 0.13 mol) was added dropwise. The reaction mixture was then warmed up to 60 °C and stirred for 2 hours at the same temperature. Then it was cooled to 25 °C and neutralized with 7 M methanolic ammonia (20 ml). The solvent and an excess of trifluoroacetic acid was removed in vacuum rotary evaporator, the residue was partitioned between ethyl acetate (200 ml) and water (200 ml). Water phase was extracted with ethyl acetate (2 x 50 ml). The combined extracts were washed with brine (30 ml), dried with anhydrous sodium sulphate and evaporated to give a crude product (foam; 12.95 g, quant.). Purity (HPLC – MS min. 93 %). The crude product was re-crystallized from ethyl acetate – petrolether (1:10); yield: 10.0 g (77.0 %). HPLC purity: 97+ %. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.98d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 3.76s (3H, CH₃), 4.02d (1H, THP), 5.63d (1H, THP), 6.66-6.70 m (1H, Ar-H), J_a=8, J_b=2.2 Hz), 7.26t (1H, Ar-H), J=8 Hz), 7.45d (H, Ar-H), J=8.0 Hz), 7.58t (1H, Ar-H), J=2.2 Hz), 8.54s (1H, C8), 10.27s (H, N6-H).

30

Table 1: Compounds Prepared by the methods of Examples 1-5



PURINE SUBSTITUENT				CHN ANALYSES	MS ANALYSES-ZMD	
	R6	R2	Cyc	[%]	[M-H] ⁻ a)	[M+H] ⁺ b)
1	anilino	chloro	tetrahydropyranyl	C=58.5; H=4.9; N=21.0	328	330
2	2-chloroanilino	chloro	tetrahydropyranyl	C=53.1; H=3.9; N=18.7	362	364
3	3-chloroanilino	chloro	tetrahydropyranyl	C=53.3; H=3.8; N=18.9	362	364
4	4-chloroanilino	chloro	tetrahydropyranyl	C=53.1; H=4.0; N=18.7	362	364
5	2-fluoroanilino	chloro	tetrahydropyranyl	C=55.4; H=4.1; N=19.7	346	348
6	3-fluoroanilino	chloro	tetrahydropyranyl	C=55.3; H=4.1; N=19.8	346	348
7	4-fluoroanilino	chloro	tetrahydropyranyl	C=55.3; H=4.1; N=19.8	346	348
8	2-hydroxyanilino	chloro	tetrahydropyranyl	C=55.9; H=4.7; N=20.0	344	346
9	3-hydroxyanilino	chloro	tetrahydropyranyl	C=56.0; H=4.5; N=19.9	344	346
10	4-hydroxyanilino	chloro	tetrahydropyranyl	C=55.9; H=4.7; N=19.8	344	346
11	2-methoxyanilino	chloro	tetrahydropyranyl	C=57.0; H=5.2; N=19.2	358	360
12	3-methoxyanilino	chloro	tetrahydropyranyl	C=56.9; H=5.2; N=19.1	358	360
13	4-methoxyanilino	chloro	tetrahydropyranyl	C=57.0; H=5.2; N=18.9	358	360
14	2-aminoanilino	chloro	tetrahydropyranyl	C=56.0; H=5.0; N=24.1	343	345
15	3-aminoanilino	chloro	tetrahydropyranyl	C=55.9; H=5.1; N=23.9	343	345
16	4-aminoanilino	chloro	tetrahydropyranyl	C=55.9; H=5.0; N=23.9	343	345
17	3,4-dimethoxyanilino	chloro	tetrahydropyranyl	C=55.9; H=5.3; N=17.6	388	390
18	2,5-dimethoxyanilino	chloro	tetrahydropyranyl	C=56.1; H=5.3; N=17.4	388	390
19	3,4,5-trimethoxyanilino	chloro	tetrahydropyranyl	C=54.9; H=5.2; N=16.0	418	420
20	3,4-dihydroxyanilino	chloro	tetrahydropyranyl	C=53.5; H=4.6; N=19.1	360	362
21	2,5-dihydroxyanilino	chloro	tetrahydropyranyl	C=53.3; H=4.5; N=19.0	360	362
22	2-chloro-5-methoxyanilino	chloro	tetrahydropyranyl	C=52.1; H=4.5; N=17.5	392	394
23	2-chloro-3-methoxyanilino	chloro	tetrahydropyranyl	C=52.0; H=4.5; N=17.8	392	394
24	2-bromo-3-methoxyanilino	chloro	tetrahydropyranyl	C=46.9; H=4.0; N=15.5	437	439
25	2-methoxy-3-chloroanilino	chloro	tetrahydropyranyl	C=52.1; H=4.6; N=17.6	392	394
26	3-ethoxyanilino	chloro	tetrahydropyranyl	C=58.1; H=5.5; N=18.6	372	374
27	2-hydroxy-5-methylanilino	chloro	tetrahydropyranyl	C=56.9; H=5.1; N=19.3	358	360
28	3-hydroxy-4-methylanilino	chloro	tetrahydropyranyl	C=57.0; H=5.1; N=19.1	358	360
29	3-hydroxy-2-methylanilino	chloro	tetrahydropyranyl	C=56.8; H=5.2; N=19.1	358	360
30	3,4-dimethylanilino	chloro	tetrahydropyranyl	C=60.8; H=5.7; N=19.1	356	358
31	2-hydroxy-3-	chloro	tetrahydropyranyl	C=54.7; H=5.0; N=18.4	374	376

	methoxyanilino					
32	2-hydroxy-4-methoxyanilino	chloro	tetrahydropyranyl	C=54.9; H=5.1; N=18.3	374	376
33	4-hydroxy-3,5-dimethoxyanilino	chloro	tetrahydropyranyl	C=53.5; H=5.1; N=16.9	404	406
34	2,3-difluoroanilino	chloro	tetrahydropyranyl	C=52.8; H=4.0; N=18.8	364	366
35	2,4-difluoroanilino	chloro	tetrahydropyranyl	C=52.8; H=3.9; N=18.7	364	366
36	2,3,4-trifluoroanilino	chloro	tetrahydropyranyl	C=50.5; H=3.5; N=17.9	382	384
37	2,4,5-trifluoroanilino	chloro	tetrahydropyranyl	C=50.3; H=3.4; N=18.0	382	384
38	2,3-dichloroanilino	chloro	tetrahydropyranyl	C=48.5; H=3.7; N=17.0	396	398
39	2,4-dichloroanilino	chloro	tetrahydropyranyl	C=48.4; H=3.6; N=17.3	396	398
40	3,5-dichloroanilino	chloro	tetrahydropyranyl	C=48.5; H=3.6; N=17.2	396	398
41	anilino	chloro	tetrahydrofuranyl	C=57.5; H=4.3; N=22.6	314	316
42	2-chloroanilino	chloro	tetrahydrofuranyl	C=51.7; H=3.5; N=19.7	348	350
43	3-chloroanilino	chloro	tetrahydrofuranyl	C=51.7; H=3.5; N=19.7	348	350
44	4-chloroanilino	chloro	tetrahydrofuranyl	C=51.9; H=3.5; N=19.5	348	350
45	2-fluoroanilino	chloro	tetrahydrofuranyl	C=54.4; H=4.0; N=20.5	332	334
46	3-fluoroanilino	chloro	tetrahydrofuranyl	C=54.1; H=3.9; N=20.3	332	334
47	4-fluoroanilino	chloro	tetrahydrofuranyl	C=54.1; H=4.0; N=20.2	332	334
48	2-hydroxyanilino	chloro	tetrahydrofuranyl	C=54.7; H=4.4; N=20.7	330	332
49	3-hydroxyanilino	chloro	tetrahydrofuranyl	C=54.9; H=4.4; N=20.6	330	332
50	4-hydroxyanilino	chloro	tetrahydrofuranyl	C=54.5; H=4.4; N=21.0	330	332
51	2-methoxyanilino	chloro	tetrahydrofuranyl	C=55.8; H=4.8; N=20.0	344	346
52	3-methoxyanilino	chloro	tetrahydrofuranyl	C=56.0; H=4.4; N=19.8	344	346
53	4-methoxyanilino	chloro	tetrahydrofuranyl	C=56.1; H=4.7; N=20.1	344	346
54	2-aminoanilino	chloro	tetrahydrofuranyl	C=54.8; H=4.7; N=25.1	329	331
55	3-aminoanilino	chloro	tetrahydrofuranyl	C=54.6; H=4.7; N=25.2	329	331
56	4-aminoanilino	chloro	tetrahydrofuranyl	C=54.9; H=4.9; N=24.9	329	331
57	3,4-dimethoxyanilino	chloro	tetrahydrofuranyl	C=54.6; H=4.9; N=18.2	374	376
58	2,5-dimethoxyanilino	chloro	tetrahydrofuranyl	C=54.9; H=5.1; N=18.1	374	376
59	3,4,5-trimethoxyanilino	chloro	tetrahydrofuranyl	C=53.9; H=5.1; N=17.0	404	406
60	3,4-dihydroxyanilino	chloro	tetrahydrofuranyl	C=53.4; H=4.6; N=19.0	346	348
61	2,5-dihydroxyanilino	chloro	tetrahydrofuranyl	C=53.6; H=4.6; N=18.8	346	348
62	2-chloro-5-methoxyanilino	chloro	tetrahydrofuranyl	C=51.0; H=4.1; N=18.0	378	380
63	2-chloro-3-methoxyanilino	chloro	tetrahydrofuranyl	C=50.9; H=4.0; N=18.2	378	380
64	2-bromo-3-methoxyanilino	chloro	tetrahydrofuranyl	C=45.5; H=3.7; N=15.9	423	425
65	2-methoxy-3-chloroanilino	chloro	tetrahydrofuranyl	C=50.7; H=4.0; N=18.0	378	380
66	3-ethoxyanilino	chloro	tetrahydrofuranyl	C=57.0; H=5.2; N=19.3	358	360

67	2-hydroxy-5-methylanilino	chloro	tetrahydrofuranyl	C=56.0; H=4.6; N=20.0	344	246
68	3-hydroxy-4-methylanilino	chloro	tetrahydrofuranyl	C=55.9; H=4.6; N=20.0	344	246
69	3-hydroxy-2-methylanilino	chloro	tetrahydrofuranyl	C=56.0; H=4.7; N=19.8	344	246
70	3,4-dimethylanilino	chloro	tetrahydrofuranyl	C=59.8; H=5.4; N=20.0	342	344
71	2-hydroxy-3-methoxyanilino	chloro	tetrahydrofuranyl	C=53.5; H=4.5; N=19.0	360	362
72	2-hydroxy-4-methoxyanilino	chloro	tetrahydrofuranyl	C=53.4; H=4.7; N=19.1	360	362
73	4-hydroxy-3,5-dimethoxyanilino	chloro	tetrahydrofuranyl	C=52.5; H=4.7; N=17.6	390	392
74	2,3-difluoroanilino	chloro	tetrahydrofuranyl	C=51.7; H=3.5; N=19.6	350	352
75	2,4-difluoroanilino	chloro	tetrahydrofuranyl	C=51.5; H=3.4; N=19.8	350	352
76	2,3,4-trifluoroanilino	chloro	tetrahydrofuranyl	C=49.0; H=3.1; N=18.8	368	370
77	2,4,5-trifluoroanilino	chloro	tetrahydrofuranyl	C=49.1; H=3.1; N=18.8	368	370
78	2,3-dichloroanilino	chloro	tetrahydrofuranyl	C=46.6; H=3.3; N=17.9	382	384
79	2,4-dichloroanilino	chloro	tetrahydrofuranyl	C=46.5; H=3.2; N=17.7	382	384

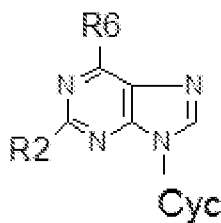
Example 6

2-Amino-6-(3-methoxytanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

To a suspension of 2-amino-6-chloro-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

- 5 (2.73 g; 0.01 mol, 2.59 g) and m-anisidine (1.23 g; 0.01 mol) in n-butanol (20 ml), triethylamine (3.5 ml; 0.025 mol) was added. The resulting thick suspension was stirred at 90°C for 5 hours. The TLC showed the absence of the starting material and the presence of the desired product. The reaction mixture was then cooled to room temperature. The white precipitate was filtered off, rinsed with n-butanol (3x10 ml) and water (3x10 ml) and dried in the drying oven into constant weight.
- 10 Yield: 2.19 g (85 %). ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.97d (2H, THP), 2.18-2.31m (1H, THP), 3.65-3.75m (1H, THP), 3.79s (3H, CH₃), 4.02d (1H, THP), 5.63d (1H, THP), 6.76dd, J_a = 8.0Hz, J_b = 2.2Hz (1H, Ar-H), 7.30t, J = 8Hz (1H, Ar-H), 7.56d, J = 8Hz (1H, Ar-H), 7.63s (1H, Ar-H), 7.66bs (2 H, NH₂), 8.34s (1H, C8-H), 11.25s (1H, N6-H).

15

Table 2: Compounds Prepared by the Method of Example 6

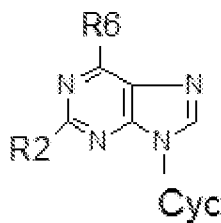
PURINE SUBSTITUENT				CHN ANALYSES	MS ANALYSES-ZMD	
	R6	R2	Cyc	[%]	[M-H] ⁻ a)	[M+H] ⁺ b)
128	anilino	amino	tetrahydropyran-2-yl	C=62.2; H=6.2; N=26.7	309	311
130	3-chloroanilino	amino	tetrahydropyran-2-yl	C=56.2; H=5.2; N=24.0	343	345
131	4-chloroanilino	amino	tetrahydropyran-2-yl	C=56.0; H=5.0; N=24.1	343	345
132	2-fluoroanilino	amino	tetrahydropyran-2-yl	C=58.9; H=5.4; N=25.2	327	329
133	3-fluoroanilino	amino	tetrahydropyran-2-yl	C=59.0; H=5.4; N=25.3	327	329
134	4-fluoroanilino	amino	tetrahydropyran-2-yl	C=59.0; H=5.4; N=25.2	327	329
135	2-hydroxyanilino	amino	tetrahydropyran-2-yl	C=59.5; H=5.8; N=25.1	325	327
136	3-hydroxyanilino	amino	tetrahydropyran-2-yl	C=59.1; H=5.6; N=25.5	325	327
137	4-hydroxyanilino	amino	tetrahydropyran-2-yl	C=59.2; H=5.6; N=25.4	325	327
138	2-methoxyanilino	amino	tetrahydropyran-2-yl	C=60.3; H=6.0; N=24.3	339	341
139	3-methoxyanilino	amino	tetrahydropyran-2-yl	C=60.2; H=6.0; N=24.2	339	341
140	4-methoxyanilino	amino	tetrahydropyran-2-yl	C=60.3; H=6.0; N=24.2	339	341
141	3,4-dimethylanilino	amino	tetrahydropyran-2-yl	C=64.0; H=6.7; N=24.4	337	339
142	anilino	amino	tetrahydropyran-2-yl	C=61.2; H=5.6; N=28.0	295	297
144	3-chloroanilino	amino	tetrahydrofuran-2-yl	C=54.6; H=4.6; N=25.0	329	331
146	2-fluoroanilino	amino	tetrahydrofuran-2-yl	C=57.7; H=4.9; N=26.5	313	315
147	3-fluoroanilino	amino	tetrahydrofuran-2-yl	C=57.7; H=4.8; N=26.5	313	315
148	4-fluoroanilino	amino	tetrahydrofuran-2-yl	C=57.4; H=4.9; N=26.4	313	315
150	3-hydroxyanilino	amino	tetrahydrofuran-2-yl	C=57.8; H=5.2; N=26.8	311	313
152	2-methoxyanilino	amino	tetrahydrofuran-2-yl	C=59.1; H=5.6; N=25.4	325	327
153	3-methoxyanilino	amino	tetrahydrofuran-2-yl	C=59.4; H=5.7; N=25.5	325	327
154	4-methoxyanilino	amino	tetrahydrofuran-2-yl	C=59.1; H=5.6; N=25.5	325	327
155	3,4-dimethylanilino	amino	tetrahydrofuran-2-yl	C=63.3; H=6.3; N=25.6	323	325

Example 7

2-Fluoro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

This compound was prepared by the reaction of 2-fluoro-6-chloro-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine (2.73 g; 0.01 mol, 2.59 g), m-anisidine (1.23 g; 0.01 mol) and triethylamine (3.5 ml; 0.025 mol) in n-butanol (20 ml) at 90 °C for 4 hours. The reaction mixture was then cooled to the room temperature. The white precipitate was filtered off, rinsed with n-butanol (3x10 ml) and water (3x10 ml) and dried in the drying oven into constant weight. Yield: 5.55 g (61 %) of yellowish crystalline powder. TLC (chloroform-methanol; 85:15): one single spot. HPLC purity: 99+ %. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.75m (1H, THP), 1.97d (2H, THP), 2.20-2.32m (1H, THP), 3.65-3.72m (1H, THP), 3.76s (3H, CH₃), 4.01d (1H, THP), 5.60d (1H, THP), 6.69dd, J_a=8.1Hz, J_b=2.2Hz(1H,Ar-H), 7.26t, J=8.1Hz(1H,Ar-H), 7.45dd, J_a=8.1Hz, J_b=2.2Hz(1H,Ar-H), 7.53t, J=2.2Hz(1H,Ar-H), 8.51s(1H,C8H), 10.36bs(1H,N6H).

Table 3: Compounds Prepared by the Method of Example 7



PURINE SUBSTITUENT				CHN ANALYSES	MS ANALYSES-ZMD	
	R6	R2	Cyc	[%]	[M-H] ⁻ a)	[M+H] ⁺ b)
80	anilino	fluoro	tetrahydropyranyl	C=61.6; H=5.2; N=22.2	312	314
81	2-chloroanilino	fluoro	tetrahydropyranyl	C=55.5; H= 4.4; N=20.0	346	348
82	3-chloroanilino	fluoro	tetrahydropyranyl	C=55.5; H= 4.4; N=20.0	346	348
83	4-chloroanilino	fluoro	tetrahydropyranyl	C=55.5; H= 4.4; N=19.8	346	348
84	2-fluoroanilino	fluoro	tetrahydropyranyl	C=58.3; H=4.7; N=20.7	330	332
85	3-fluoroanilino	fluoro	tetrahydropyranyl	C=58.2; H=4.8; N=20.9	330	332
86	4-fluoroanilino	fluoro	tetrahydropyranyl	C=58.3; H=4.7; N=20.8	330	332
87	2-hydroxyanilino	fluoro	tetrahydropyranyl	C=58.5; H=5.1; N=20.9	328	330
88	3-hydroxyanilino	fluoro	tetrahydropyranyl	C=58.7; H=5.2; N=21.0	328	330
89	4-hydroxyanilino	fluoro	tetrahydropyranyl	C=58.6; H=5.2; N=21.0	328	330
90	2-methoxyanilino	fluoro	tetrahydropyranyl	C=59.7; H=5.4; N=20.1	342	244
91	3-methoxyanilino	fluoro	tetrahydropyranyl	C=59.9; H=5.6; N=19.9	342	244
92	4-methoxyanilino	fluoro	tetrahydropyranyl	C=59.7; H=5.5; N=20.0	342	244
93	3,4-dimethoxyanilino	fluoro	tetrahydropyranyl	C=58.4; H=5.5; N=18.3	372	374
94	2,5-dimethoxyanilino	fluoro	tetrahydropyranyl	C=58.6; H=5.4; N=18.4	372	374
95	3,4-dihydroxyanilino	fluoro	tetrahydropyranyl	C=55.8; H=4.8; N=19.9	344	346
96	2,5-dihydroxyanilino	fluoro	tetrahydropyranyl	C=55.7; H=4.7; N=20.0	344	346
97	2-chloro-5-methoxyanilino	fluoro	tetrahydropyranyl	C=54.2; H=4.7; N=18.2	376	378
98	2-methoxy-3-chloroanilino	fluoro	tetrahydropyranyl	C=54.3; H=4.7; N=18.2	376	378
99	3-ethoxyanilino	fluoro	tetrahydropyranyl	C=60.6; H=5.7; N=19.2	356	358
100	2-hydroxy-5-methylanilino	fluoro	tetrahydropyranyl	C=59.7; H=5.4; N=20.2	342	344
101	3-hydroxy-4-methylanilino	fluoro	tetrahydropyranyl	C=59.6; H=5.4; N=20.1	342	344
102	3-hydroxy-2-methylanilino	fluoro	tetrahydropyranyl	C=59.7; H=5.4; N=20.1	342	344
103	3,4-dimethylanilino	fluoro	tetrahydropyranyl	C=62.6; H=6.1; N=20.4	340	342
104	anilino	fluoro	tetrahydrofuranyl	C=59.9; H=4.9; N=23.2	298	300

106	3-chloroanilino	fluoro	tetrahydrofuranyl	C=54.3; H= 4.1; N=20.8	332	334
109	3-fluoroanilino	fluoro	tetrahydrofuranyl	C=57.0; H=4.3; N=21.7	316	318
112	3-hydroxyanilino	fluoro	tetrahydrofuranyl	C=57.4; H=4.6; N=21.9	314	316
114	2-methoxyanilino	fluoro	tetrahydrofuranyl	C=58.8; H=5.1; N=21.0	328	330
115	3-methoxyanilino	fluoro	tetrahydrofuranyl	C=58.7; H=5.1; N=21.1	328	330
116	4-methoxyanilino	fluoro	tetrahydrofuranyl	C=58.7; H=5.2; N=21.1	328	330
117	3,4-dimethoxyanilino	fluoro	tetrahydrofuranyl	C=58.2; H=5.5; N=18.5	358	360
120	2,5-dihydroxyanilino	fluoro	tetrahydrofuranyl	C=54.5; H=4.4; N=20.8	330	332
121	2-chloro-5-methoxyanilino	fluoro	tetrahydrofuranyl	C=53.1; H=4.2; N=19.0	362	364
122	2-methoxy-3-chloroanilino	fluoro	tetrahydrofuranyl	C=53.0; H=4.3; N=19.1	362	364
123	3-ethoxyanilino	fluoro	tetrahydrofuranyl	C=59.6; H=5.4; N=20.1	342	344
124	2-hydroxy-5-methylanilino	fluoro	tetrahydrofuranyl	C=58.5; H=5.0; N=21.0	328	330
125	3-hydroxy-4-methylanilino	fluoro	tetrahydrofuranyl	C=58.4; H=5.0; N=20.9	328	330
126	3-hydroxy-2-methylanilino	fluoro	tetrahydrofuranyl	C=58.5; H=5.0; N=20.9	328	330
127	3,4-dimethylanilino	fluoro	tetrahydrofuranyl	C=62.4; H=5.6; N=21.5	326	328

Example 8

2-Methylthioxanthine

Dimethyl sulfate (77 g, 58 ml) was added dropwise into a freshly prepared solution of 2-thioxanthine (103g, 0.613 mol) in 2 M NaOH (613 ml) and water (245 ml), temperature was maintained between 25 and 40 °C. Reaction mixture was stirred for another hour and then left to stand overnight at room temperature. The dark red liquid was then filtered and the product was precipitated by concentrated acetic acid, filtered and re-crystallized from hot water (1500 ml) with active carbon. The product was crystallized at -10 °C, filtered and dried at 95 °C for 3 h, and then over phosphorus(V) oxide to constant weight. Yield: 48 %. HPLC purity: 80 %.

Example 9

2-Methylthio-6-chloropurin

2-Methylthioxanthine (65 g) was mixed with POCl₃ (975 ml) and N,N-diethylaniline (97.5 ml). Reaction mixture was then refluxed at mechanical stirring for 90 minutes. Excess POCl₃ was removed by vacuum rotary evaporator at 55-60 °C and the residue was mixed with ice (0 °C, 1.75 kg) for 10 minutes (until total hydrolysis of POCl₃ was achieved). The product was then extracted by ethyl acetate (4 x 2.5 l). The extracts were washed with water and evaporated to dryness, yielding a dark brown solid. The raw product was re-crystallized from ethanol with active carbon

and dried to constant weight over phosphorus(V) oxide. Yield: 23.6 g (33%). HPLC purity: 99%. TLC (chloroform/methanol, 9/1) – no impurities.

Example 10

5 *2-Methylthio-6-anilinopurine*

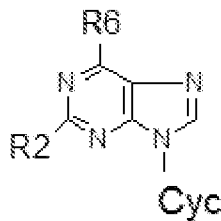
2-methylthio-6-chloropurine (10 g, 50 mmol), aniline (7.7 g, 63 mmol) and triethylamine (35 ml, 250 mmol) were refluxed in butanol (400 ml) for 2 h. Butanol was removed in vacuo and water (290 ml) added to the cooled residue. pH was adjusted to 8.0, the mixture was left at -16 °C overnight. Filtration and drying over phosphorus pentoxide in vacuo gave 10 g crude yellow-green
10 product. The product was chromatographically purified (250 g silica, Fisons, chloroform); eluted with chloroform/methanol (97/3, v/v). Appropriate fractions were dried in vacuo, re-crystallised from ethanol with decolourisation by active carbon. After drying to constant weight over P₂O₅, yield 5.76 g, (46%), HPLC purity >98%. TLC: chloroform/methanol (9/1, v/v), no contaminants.

15 Example 11

2-Methylthio-6-anilino-9-(tetrahydropyran-2-yl)purin

3,4-dihydropyran (10 ml, 0.1 mol) and trifluoroacetic acid (10 ml, 0.13 mol) were added in this order to the suspension of 2-methylthio-6-anilinopurine (10 g; 0.039 mol) in dioxane (250 ml). The reaction mixture was then heated to 60 °C, and stirred at this temperature for 2 hours. Then the
20 reaction mixture was cooled to 25 °C and neutralized with 7 M methanolic ammonia at cooling. Dioxane was distilled off under vacuum and the residue was worked up with ethyl acetate (200 ml) and water (200 ml). Aqueous phase was extracted by ethyl acetate (2 x 50 ml). Combined organic extracts were shaken with saline (30 ml), dried with dehydrated sodium sulfate and evaporated on rotary vacuum evaporator. Yield: 13.4 g (quant.) of beige amorphous product. HPLC purity: 93 %
25 +. The raw product was re-crystallized from the mixture ethyl acetate – petrolether (1 : 10); yield: 10.3 g (77.0 %) of off-white product. HPLC purity: 97 % +. ¹H-NMR, DMSO-d₆, TMS, ppm: 1.58m (2H, THP), 1.72-1.78m (1H, THP), 1.98d (2H, THP), 2.18-2.31m (1H, THP), 2.49s (3H, CH₃S), 3.65-3.75m (1H, THP), 4.02d (1H, THP), 5.63d (1H, THP), 6.69dd, J_a=8.2Hz, J_b=2.2Hz(1H,Ar-H), 7.29t, J=8.2Hz, 1H(Ar-H), 7.46dd, J_a=8.2Hz, J_b=2.2Hz(1H,Ar-H), 7.67t, J=2.2Hz(1H,Ar-H), 9.00s(1H,C8H), 10.74s(1H,N6H).
30

Table 5: Compounds Prepared by the Method of Examples 8-11



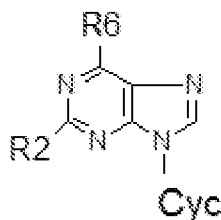
PURINE SUBSTITUENT				CHN ANALYSES	MS ANALYSES-ZMD	
	R6	R2	Cyc	[%]	[M-H] ⁻ a)	[M+H] ⁺ b)
157	anilino	methylthio	tetrahydropyranyl	C=60.0; H=5.7; N=20.3	340	342
158	3-chloroanilino	methylthio	tetrahydropyranyl	C=54.6; H=4.9; N=18.2	374	376
159	3-fluoroanilino	methylthio	tetrahydropyranyl	C=56.9; H=5.1; N=19.4	358	360
160	4-fluoroanilino	methylthio	tetrahydropyranyl	C=56.9; H=5.2; N=19.4	358	360
161	3-hydroxyanilino	methylthio	tetrahydropyranyl	C=57.5; H=5.5; N=19.2	356	358
162	2-methoxyanilino	methylthio	tetrahydropyranyl	C=58.5; H=5.7; N=18.6	370	372
163	3-methoxyanilino	methylthio	tetrahydropyranyl	C=58.4; H=5.8; N=18.7	370	372
164	4-methoxyanilino	methylthio	tetrahydropyranyl	C=58.5; H=5.8; N=18.7	370	372
165	3,4-dimethoxyanilino	methylthio	tetrahydropyranyl	C=56.5; H=5.9; N=17.2	400	402
166	2,5-dimethoxyanilino	methylthio	tetrahydropyranyl	C=56.7; H=5.1; N=17.4	400	402
167	2-chloro-5-methoxyanilino	methylthio	tetrahydropyranyl	C=53.5; H=5.1; N=17.0	404	406
168	2-methoxy-3-chloroanilino	methylthio	tetrahydropyranyl	C=53.6; H=5.1; N=17.1	404	406
169	anilino	methylthio	tetrahydrofuranyl	C=58.9; H=5.3; N=21.1	326	328
170	2-methoxyanilino	methylthio	tetrahydrofuranyl	C=57.4; H=5.5; N=19.2	356	358
171	3-methoxyanilino	methylthio	tetrahydrofuranyl	C=57.1; H=5.5; N=19.4	356	358
172	4-methoxyanilino	methylthio	tetrahydrofuranyl	C=57.2; H=5.4; N=19.3	356	358

Example 12

5 2-Bromo-6-(methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine

2-Bromo-6-(methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine was prepared in a similar manner to example 5 by the reaction of 2-bromo-6-chloro-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine (2.73 g; 0.01 mol, 2.59 g) and m-anisidine (molar ratio 1:1) in the presence of triethylamine (2.5 eq.) in n-propanol at 90 °C for 5 hours. After cooling to room temperature the resulting white precipitate was filtered off, washed with cold n-propanol and water and dried in desiccator into constant weight. Yield: 69%. Crude product was purified by crystallization from methanol and free base was obtained by treatment of hydrobromide with 10% aqueous ammonia. HPLC purity: 98+%.

10

Table 6: Compounds Prepared by the Method of Example 12

PURINE SUBSTITUENT				CHN ANALYSES	MS ANALYSES-ZMD	
	R6	R2	Cyc	[%]	[M-H] ⁻ a)	[M+H] ⁺ b)
173	Anilino	bromo	tetrahydropyranyl	C=51.6; H=4.4; N=21.1	373	375
174	2-chloroanilino	bromo	tetrahydropyranyl	C=47.4; H=3.8; N=16.9	407	409
175	3-chloroanilino	bromo	tetrahydropyranyl	C=47.2; H=3.8; N=17.0	407	409
176	2-fluoroanilino	bromo	tetrahydropyranyl	C=49.3; H=4.0; N=17.7	391	393
177	3-fluoroanilino	bromo	tetrahydropyranyl	C=49.1; H=3.9; N=17.6	391	393
178	3-methoxyanilino	bromo	tetrahydropyranyl	C=50.7; H=4.6; N=17.0	403	405
179	3-hydroxyanilino	bromo	tetrahydropyranyl	C=49.5; H=4.2; N=17.9	389	391
180	Anilino	bromo	tetrahydrofuranyl	C=50.2; H=4.0; N=19.1	359	361
181	3-chloroanilino	bromo	tetrahydrofuranyl	C=45.8; H=3.3; N=17.6	393	395
182	2-fluoroanilino	bromo	tetrahydrofuranyl	C=48.1; H=3.6; N=18.2	377	379
183	3-fluoroanilino	bromo	tetrahydrofuranyl	C=48.0; H=3.5; N=18.3	377	379
184	3-methoxyanilino	bromo	tetrahydrofuranyl	C=49.6; H=4.3; N=17.6	389	391
185	3-hydroxyanilino	bromo	tetrahydrofuranyl	C=48.0; H=3.8; N=18.5	375	377

5 Example 13

Agonistic activity of 2-chloro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl)purine (compound **12**) and 2-fluoro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl)purine (compound **91**) on cytokinin receptors

Escherichia coli strains KMI001 harbouring the plasmid pIN-III-AHK4 or pSTV28-AHK3 were grown overnight at 25 °C in M9 media enriched with 0.1% casamino acids to OD₆₀₀~1. The preculture was diluted 1:600 in 1 ml M9 medium containing 0.1% casamino acids and 1 µL stock solution of either the tested compound (10⁻⁷ M – 5 x 10⁻⁵ M) or solvent control (DMSO, ethanol, methanol) were added. The cultures were further grown at 25 °C in microtiter plate, 200 µL per well. Incubation times of 17 h and 28 h were found to be optimal for CRE1/AHK4 and AHK3, respectively. The cultures were centrifuged and 50 µL aliquots of the supernatant were transferred to microtiter plate containing 2 µL 50 mM 4-methyl umbelliferyl galactoside which was subsequently incubated for 1 h at 37 °C. The reaction was stopped by adding 100 µL 0.2 M Na₂CO₃. Fluorescence was measured using a Fluoroscan Ascent (Labsystems, Finland) at the

excitation and emission wavelengths of 365 and 460 nm, respectively. The OD₆₀₀ of remaining culture was determined and β -galactosidase activity was calculated as nmol 4-methylumbelliferone x OD₆₀₀⁻¹ x h⁻¹.

The curves shown in Figure 1 show that compared to the cytokinin *trans*-zeatin (tZ) the novel compound **12** have very low ability to activate the cytokinin receptors AHK3 and CRE1/AHK4, and compound **91** do not activate any of the receptors. This indicates that if a cytokinin-like activity is observed after application of the compounds **12** and **91**, it is resulting from mechanism different from direct activation of the cytokinin receptor pathway. The comparison with structurally related CKX inhibitors INCYDE (2-chloro-6-(3-methoxyanilino)purine) and INCYDE-F (2-fluoro-6-(3-methoxyanilino)purine) also indicates that the novel compounds have original mode of action different from the CKX inhibitors INCYDE and INCYDE-F.

Example 14

Induction of cytokinin reporter ARR5 expression after application of 2-chloro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl)purine (compound 12)

Cytokinins induce in *Arabidopsis* transcription of gene *ARR5*, a member of A-type response regulator family classified as cytokinin primary response genes being expressed down-stream of sensing by cytokinin receptors. We cultivated transgenic *Arabidopsis* plants harbouring *P_{ARR5}::GUS* reporter (D'Agostino et al. Plant Physiol. 124: 1706-1717, 2000) in MS medium containing cytokinin BAP, CKX inhibitor INCYDE and novel compounds **12** and **91** in concentrations 0.01, 0.1, 1, 5, 10 μ M; DMSO (0.1%) was tested as solvent control. Seeds were surface-sterilized in 70% ethanol and then placed into wells of 6-well microtiter plate (TPP, Switzerland) containing 3 mL of MS medium in each well. After sowing plates were pretreated in dark at 4°C to synchronize the germination of the seeds. Then the plates were transferred to growth chamber (22 °C, 16h light/8h dark) and after 2-3 days after germination the tested compounds were applied directly into the media. The plants were incubated with the tested compounds for 17 h. Quantitative estimation of the level of *ARR5::GUS* gene induction was done according to method published by Romanov et al. (Romanov et al. FEBS Letters 515: 39-43, 2002). After extraction of proteins the GUS activity was determined using incubation with fluorogenic substrate MUG (4-methylumbelliferyl glucuronid; 1 h, 37 °C) and then fluorescence 365 nm and 460 nm (excitation and emission wavelengths) was recorded, as described in detail by Spíchal et al. (Spíchal et al. Plant and Cell Physiology 45: 1299-1305, 2004).

Quantitative assay showed compound **12** and **91** dose-dependent induction of *ARR5::GUS* expression (Fig. 2). Taking into account that the compound **12** activated cytokinin receptors only weakly and compound **91** fails to activate cytokinin receptors at all (see Fig. 1 from previous example), it can be concluded that these compounds do not act as cytokinins directly, but after their application to the plants cytokinin pathway is activated indirectly. Comparison with activities of

CKX inhibitors INCYDE (2-chloro-6-(3-methoxyanilino)purine) and INCYDE-F (2-fluoro-6-(3-methoxyanilino)purine) (Fig 2) also documents that compounds **12** and **91** have different mode of action than the mentioned structurally related CKX inhibitors.

5 Example 15

Inhibition of cytokinin oxidase/dehydrogenase activity

IC₅₀ measurements were done using the assay in microtitre plate. Each well contained 100 µL of PMS/MTT [phenazine methosulfate/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reaction mixture (final concentrations: 0.1 M KH₂PO₄, pH 7.4, 1 mM MTT, 0.2 mM PMS) containing the tested compound (3 x 10⁻⁷ M – 3 x 10⁻⁴ M) and 30 µM N⁶-isopentenyladenine (iP) as a substrate. 100 µL of cell-free growth medium of *S. cerevisiae* strain 23344c *ura*⁻ harboring the plasmid pYES2-AtCKX2 was directly used as a source of AtCKX2. Plates were incubated in the dark for 30 min at 37 °C and the enzymatic reaction was stopped by 25 µL of 35% acetic acid. The absorbance at 578 nm was measured using spectrophotometer Tecan. Absorbance of the sample without iP was subtracted.

The IC₅₀ value, the compound concentration that inhibits the enzyme activity to 50 %, was calculated from the obtained dose response curves. The values shown in Table 7 are means of three replicates and the entire test was repeated at least twice. More potent compounds than thidiazuron have IC₅₀ values lower than thidiazuron. Compound **12** is an effective inhibitor of AtCKX2 activity, app. 3-times stronger than thidiazuron and only app. 3-times weaker than the structurally related compound INCYDE (2-chloro-6-(3-methoxyanilino)purine). It indicates that compound **12** can directly act as an original inhibitor of CKX activity in plant tissues and thus increase the endogenous cytokinin levels. On the other hand the visible difference in activity of compound **91** and its structurally related CKX inhibitor INCYDE-F (2-fluoro-6-(3-methoxyanilino)purine) indicates that biological activity of compound **91** is caused also by other mechanism different from CKX inhibition.

Table 7: The effect of novel compounds on inhibition of recombinant AtCKX2

No.	Tested compound			IC ₅₀ (µmol.L ⁻¹)
	R6	R2	R9	
	thidiazuron			55
	INCYDE			6.7
	INCYDE-F			5.2
12	3-methoxyanilino	chloro	tetrahydropyr	19.5
91	3-methoxyanilino	fluoro	tetrahydropyr	380

Example 16

In vitro effect of 2-chloro-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl) purine (compound 12) on shoot and root growth of cytokinin deficient Arabidopsis plants

- 5 To prove that compound **12** has positive effect on plant growth and development through inhibition of the cytokinin degradation enzyme CKX (thus through stabilisation of plant endogenous cytokinin levels) and that the phenotype complementation effect is not general effect of exogenously applied cytokinin, CKX1 overproducing (35S:CKX1) *Arabidopsis* seedlings were grown *in vitro* on a standard MS medium containing 0.1 μ M cytokinin BAP, the same
- 10 concentration of compound **12** and the same concentration of CKX inhibitor INCYDE (2-chloro-6-(3-methoxyanilino)purine). Figure 3 clearly shows that the application of compound **12** as well as CKX inhibitor INCYDE released the plants from growth inhibition caused by decreased cytokinin level and led to restauration of wild type shoot phenotype in a dose dependent manner (Fig 4). Such an effect cannot be reached by application of the cytokinin BAP (WO 2008/CZ00074).
- 15 To further prove that the action of the compound **12** is different from structurally similar CKX inhibitor INCYDE (2-chloro-6-(3-methoxyanilino)purine), effect of the both compounds on the root growth of the 35S:CKX1 *Arabidopsis* plants was assessed. As shown in Figure 5 whereas INCYDE did not influence the root biomass in the tested concentration range, compound **12** exert strong dose-dependent positive effect. Constitutive overexpression of CKX in 35S:CKX1 plants
- 20 leads to enhanced root growth. If CKX inhibitor can restore WT phenotype, it is expected that inhibition of the CKX activity in 35S:AtCKX1 plants should thus have no effect or inhibit their root growth. Such observation was done in case of INCYDE, but not compound **12**. Thus, despite the fact that compound **12** is capable of inhibition of CKX activity (example 16) the result described in this example clearly indicates that the biological activity of compound **12** is different
- 25 from specific CKX inhibitor INCYDE.

Example 17

In vitro effect of 2-chloro-6-(3-methoxyanilino)-9-tetrahydropyran-2-ylpurine (compound 12) on root growth of wild-type Arabidopsis plants

- 30 As shown in previous example presence of compound **12** in the growth medium has positive effect on the root growth of cytokinin-deficient *Arabidopsis* plants. This effect was further studied using wild-type *Arabidopsis* plants and compared with biologically and structurally relevant compounds, a cytokinin BAP and a CKX inhibitor INCYDE. 35S:CKX1 *Arabidopsis* seedlings were grown *in vitro* on a standard MS medium containing 0.1 μ M cytokinin BAP, the same concentration of
- 35 compound **12** and the same concentration of CKX inhibitor INCYDE (2-chloro-6-(3-methoxyanilino)purine). Figure 6 clearly shows that whereas cytokinin BAP and CKX inhibitor

INCYDE expectedly inhibited root growth, application of compound **12** showed positive effect on the root length and lateral branching.

Example 18

5 *Effect of 2-chloro-6-(3-methoxyanilino)aminopurine (compound 12) on leaf chlorophyll degradation in shoot rosettes of Arabidopsis*

The effect of the compound **12** on retention of chlorophyll in the dark treated plants was tested using app. 18 DAG (days after germination) old Arabidopsis plants grown in vitro on standard MS medium (16h/8h light/dark regime, light intensity $120 \mu\text{mol.m}^{-1}.\text{s}^{-1}$). Shoots of the plants were
10 excised from the roots and the whole rosettes were placed into 6-well plate (one rosete per well) into water (3 mL per well) containing 0.01% DMSO (solvent control), or compound **12**, cytokinin BAP and CKX inhibitor INCYDE (2-chloro-6-(3-methoxyanilino)purine), in concentrations of 0.01, 0.1, 1 and $10 \mu\text{M}$. The plates were then incubated in the dark for 6 days at 25°C . After
15 incubation chlorophyll from each rosette was extracted into 1.8 mL of 80% ethanol, during 30 min of heating to 80°C . The chlorophyll content in extracts was measured spectrophotometrically at 665 nm. As shown in Figure 7, compound **12** showed highest activity in $1 \mu\text{M}$ concentration exceeding that of both cytokinin BAP and CKX inhibitor INCYDE. In this concentration the rosettes kept around 95% of chlorophyll.

20 Example 19

Effect of drenching application of compound 12 on morphological and yield traits of Arabidopsis

Presence of the compound **12** in the growth medium of *in vitro* grown cytokinin-deficient Arabidopsis plants showed positive effect on both shoot and root growth (see Example 22 and 23). To test the effect on wild-type plants *Arabidopsis* (ecotype Col-0) was grown in soil in controlled
25 conditions in a growth chambre (16 hours light/8 hours dark; light intensity $120 \mu\text{mol.m}^{-1}.\text{s}^{-1}$; 22°C ; 60% humidity). In the stage of app. the middle of the principal growth stage 1 and the beginning of principal growth stage 5 (according to Arabidopsis growth stages described by Boyes et al., 2001) 1-10 mL of the compound **12** was applied regularly every third day in 0.1 and $1 \mu\text{M}$ concentration in different application rates (experiment 1 – 1 mL of $1 \mu\text{M}$ compound 12 water solution 12-times;
30 experiment 2 - 10 mL of 0.1 and $1 \mu\text{M}$ compound 12 water solution 1-, 3- and 5-times) by drenching to each plant pot. DMSO (0.01%) was used as a solvent control. The plants were grown till the end of the generative stage and then morphological parametres (primary inflorescence stem length, number of auxiliary branches, number of of lateral branches, fresh shoot biomass, number of flowers, number of siliques) and the seed yield were recorded.

35 The initial experiment 1 showed that the regular drenching with $1 \mu\text{M}$ compound **12** leads to stimulation of the overall shoot biomass (Fig 8). In the subsequent experiment 2 effect of drenching

with two concentrations (0.1 and 1 μM) of compound **12** was tested in three application rates (1x, 3x, 5x). All the applications had stimulatory effect on the overall shoot biomass and significantly increased the monitored morphological parameters (Fig. 9). As summarized in Table 8 the higher doses increased the primary inflorescence stem length by 16-34%, number of auxiliary branches by 6-25%, number of lateral branches by 77-249%, fresh shoot biomass by 6-22%, number of flowers by 20-89%, number of siliques by 5-66%, and the seed yield by 6-26%, compared to control, respectively (for particular values see Table 8).

The presented data indicate that drenching application with compound **12** increases significantly plant performance leading to increase in shoot architecture, biomass and total seed yield of *Arabidopsis* model plant.

Example 20

Effect of seed coating with compound 12 on shoot and root growth of maize (pot experiment)

The drenching experiments with model plant *Arabidopsis* pointed to potential application of compound **12** in stimulation of plant growth and development when the roots of the plants are exposed to the chemical. To prove this effect of compound **12** applied by seed coating was tested using maize. In the year 2011 pot experiment was performed with maize (hybrid Amadeo; KWS, FAO 230). The seeds were coated with 10 and 50 μM concentration of compound **12** mixed into seed treatment agent Cruiser OSR (Syngenta; 8 L of solution per 1000 kg of seeds). Seeds coated with only Cruiser OSR were used for control variant. The seeds were dried and then sown into the soil, one plant per 15 L pot (30 plants were used per variant). The plants were harvested after approximately one month in stage DC 18 (eight leaves per plant). The shoots and the roots of each plant were separated and dried. The weight of dry shoot and root biomass was recorded. As shown in Figure 10 compound **12** showed dose-dependent stimulatory effect on the both shoot and root dry biomass. The plants, which seeds were coated with the lower concentration of compound **12**, formed 2-times higher root dry biomass and 2.75-times higher shoot dry biomass. In total this was reflected by 2.3-times increase of the total plant dry weight.

This data indicate that compound **12** can be used with advantage for seed applications to improve the biomass of crops such as maize.

Example 21

Effect of seed coating with compound 12 on shoot biomass of silage maize (field experiment)

Based on the results discussed in previous Example 25, field plot experiments were performed on two different locations (Kroměříž, Czech Republic; Olomouc, Czech Republic) in years 2012 and 2013 to evaluate the effect of seed coating with compound **12** on biomass formation of maize. In

Table 8. Phenotypic analysis of *Arabidopsis* plants treated with compound 12. The experiment was repeated three times with comparable results. In each experiment 30 plants per each variant were used. Stars indicate * $P < 0.05$, * $P < 0.01$, *** $P < 0.001$ (Student's t-test, $n = 30$).

Trait	Control	0.1 μM			1 μM		
		application rate 1x	application rate 3x	application rate 5x	application rate 1x	application rate 3x	application rate 5x
primary inflorescence stem length (cm)	33,26 $\pm$ 3,4	32,78 $\pm$ 5,5 (98%)	39,6 $\pm$ 3,8 (119%)***	38,61 $\pm$ 5,2 (116%)***	32,66 $\pm$ 3,8 (98%)	39,07 $\pm$ 3,2 (117%)***	44,66 $\pm$ 5,9 (134%)***
No. of auxiliary branches	3,59 $\pm$ 0,7	4,06 $\pm$ 1,3 (113%)	3,82 $\pm$ 0,6 (106%)	3,94 $\pm$ 1,1 (109%)	4,29 $\pm$ 1,5 (119%)	4,29 $\pm$ 1,1 (119%)*	4,50 $\pm$ 1,3 (125%)
No. of lateral branches	4,71 $\pm$ 1,8	8,59 $\pm$ 3,1 (182%)***	8,35 $\pm$ 3,3 (177%)***	9,82 $\pm$ 4,4 (208%)***	13,35 $\pm$ 4,7 (283%)***	13,50 $\pm$ 3,9 (286%)***	16,44 $\pm$ 6,1 (349%)***
No. of flowers	75,71 $\pm$ 27,8	116,12 $\pm$ 70 (153%)*	91,23 $\pm$ 50,1 (120%)	99,59 $\pm$ 59,3 (131%)	139,65 $\pm$ 51,7 (184%)***	118,53 $\pm$ 36,6 (156%)***	143,56 $\pm$ 66,3 (189%)***
No. of siliques	133,59 $\pm$ 33,8	133,94 $\pm$ 47 (100%)	154,29 $\pm$ 25,6 (115%)	141,24 $\pm$ 31,7 (105%)	134,82 $\pm$ 31,1 (100%)	155,29 $\pm$ 44,1 (116%)	220,00 $\pm$ 37,4 (164%)***
fresh shoot biomass (g)	1,09 $\pm$ 0,3	1,18 $\pm$ 0,4 (108%)	1,16 $\pm$ 0,2 (106%)	1,18 $\pm$ 0,3 (108%)	1,17 $\pm$ 0,3 (107%)	1,23 $\pm$ 0,3 (113%)	1,81 $\pm$ 0,3 (166%)***
seed yield (g) per plant	0,29 $\pm$ 0,2	0,30 $\pm$ 0,1 (102%)	0,31 $\pm$ 0,1 (106%)	0,35 $\pm$ 0,1 (120%)	0,37 $\pm$ 0,1 (126%)	0,36 $\pm$ 0,1 (122%)	0,33 $\pm$ 0,1 (111%)

the season 2012 the seeds of maize (hybrid Amadeo; KWS, FAO 230) were coated with 10 and 50 μM water solution of compound **12** (8 L of solution per 1000 kg of seeds). The seeds were dried and then sown in the sowing rate 90.000 seeds per hectare in 4 replications per each variant in randomized positions (each replication was 10 square meter plot). The plants were harvested when reached the stage of 33-34 % of dry mass. The plants were then analyzed for their moisture and standardized dry mass.

The experiment was repeated in the season 2013 with 50 μM concentration of compound **12** in the same experimental design with maize hybrid Zidane (KWS, FAO 250).

The results obtained in all field plot experiments are summarized in Table 9. The data show that the seed treatment with compound **12** positively influenced the dry biomass of maize without any effect on the plant height. The average silage yield counted from all trials performed shows 110,9% increase in case of concentration 10 μM (counted from 2 trials) and 105,3 % in case of concentration 50 μM (counted from 3 trials), respectively. This data indicate that compound **12** can be used with advantage for seed applications to improve the biomass of crops such as maize in field conditions.

Table 9. Summary of effects of on-seed application of compound **12** on maize silage yield obtained in field plot experiments performed at two localities in years 2012 and 2013 with maize hybrids Amadeo (KWS, FAO 230) and Zidane (KWS, FAO 250). Average silage yield of control was 50.95 t/ha (in Olomouc), and 49.60 t/ha (in Kroměříž), respectively. Asterics indicate P value 0,01 – 0,05.

Variant	Absolute green matter / dry matter yield	Relative dry matter yield (% of control)	Height before harvest (cm / % of control)	Location, year
Cpd 12 (50 μM)	68.78 / 19.23	101.33	246.4 / 100.8	Olomouc, 2013
	66.47 / 22.33	107.82 *	-	Kroměříž, 2013
Cpd 12 (10 μM)	43.20 / 17.01	110.94	237.3 / 98.3	Olomouc, 2012
Cpd 12 (50 μM)	44.31 / 17.80	116.06	235.8 / 97.7	Olomouc, 2012
Cpd 12 (10 μM)	52.19 / 23.06	110.85	276.6 / 101.0	Kroměříž, 2012
Cpd 12 (50 μM)	49.52 / 22.18	106.63	273.5 / 99.8	Kroměříž, 2012

Example 22

*Effect of seed coating with compound **12** on tillering of winter wheat (field experiment)*

To evaluate the effect of seed coating with compound **12** on morphological yield forming traits of winter wheat field plot experiment was performed in year 2012 on locality Olomouc, Czech Republic. Seeds of winter wheat variety Diadem (Selgen) were coated with 10 and 50 μM concentration of compound **12** mixed into seed treatment agent Cruiser 350FS (Syngenta; 10 L of solution per 1000 kg of seeds), seeds coated with only Cruiser 350FS were used for control variant.

The seeds were dried and then sown in the sowing rate 3.500.000 seeds per hectare in 4 replications per each variant in randomized positions (each replication was 10 square meter plot). The plants were analyzed for number of productive tillers, medium tillers and weak tillers. The data summarized in Table 10 show that seed treatment with compound **12** led to significant dose-dependent increase in number of productive tillers and reduction of medium and weak tillers, respectively. The number of productive tillers was increased to 106 – 124% compared to untreated control.

This data indicate that compound **12** can used with advantage for seed applications to improve the tillering as an important yield forming trait of cereals such as winter wheat in field conditions.

Table 10. Summary of effect of seed application of compound 12 on tillering of winter wheat. Asterics indicate significant differences, ** $P \geq 0.01$ and * $P = 0.01 - 0.05$.

Variant	Number of productive tillers (% of control)	Number of medium tillers (% of control)	Number of weak tillers (% of control)	Location, harvest year
Control	2.33 tillers	1.60 tillers	1.15 tillers	Olomouc, 2012
Cpd 12 (10 μ M)	106.5	75.0 *	102.2	Olomouc, 2012
Cpd 12 (50 μ M)	124.2 **	78.1 *	80.4	Olomouc, 2012

Example 23

Effect of seed coating with compound 12 on emergence and freezing tolerance of winter rapeseed (field experiment)

To evaluate the effect of seed coating with compound **12** on yield forming traits of winter rapeseed field plot experiment was performed in season 2010/2011 on locality Opava, Czech Republic. Seeds of winter rapeseed variety Benefit (Selgen) were coated with 50 μ M concentration of compound **12** mixed into the seed coating treatment Cruiser OSR (Syngenta; 15 L of solution per 1000 kg of seeds), seeds coated with only Cruiser OSR were used for control variant. The seeds were dried and then sown in the sowing rate 700.000 seeds per hectare in 4 replications per each variant in randomized positions (each replication was 10 square meter plot). In the experiment plant emergence, freezing tolerance and final seed yield were scored. The frost tolerance was estimated by method described in (Janáček, J., Prášil, I.: Quantification of plant frost injury by nonlinear fitting of an s-shaped function. Cryo-Letters vol.12, 1991, no.7, 47-52).

The results summarized in Table 13 show that seed application of compound **12** stimulated plant emergence (app. 20% more plants emerged in the same time compared to control). The treatment significantly decreased leaf biomass compared to control, whereas it had slightly stimulatory effect on the root length, leading to improved ratio between shoot and root towards the root system. The artificially estimated frost tolerance was significantly increased app. by 5%. The treatment in this trial increased the final seed yield by 3.1%.

This data indicate that compound **12** can be used with advantage for seed applications to improve the winter tolerance and subsequent final seed yield of winter rapeseed in field conditions.

- Table 11. Summary of effect of seed application of compound 12 on yield influencing traits and final seed yield of winter rapeseed. Seed yield was counted as mean from 4 plots, other traits were counted from 15 randomly chosen plants. Asterics indicate significant differences, ** $P \geq 0.01$ and * $P = 0.01 - 0.05$.

Trait	control	Cpd 12 (50 μ M)
Seed yield (t / ha)	5.80	5.98
Seed yield (% of control)	100	103.1
Leafs weight – autumn (22th October)	17,24	11,69 **
Root lenght (cm) – autumn (22th October)	14,03	14,63
Number of emerged plants per sq meter – autumn (11th October, 2010)	49.5	60.0
Plant height (cm) - autumn (11th October, 2010)	6.0	6.6
Estimated frost resistance (atrifical frost test) – Letal Temperature LT50	-11.7	-12.3 *

Example 24

10 *In vitro* cytotoxic activity of novel compounds

Absence of toxic effects against mammalian (especially human) cell lines in a wide concentration range is one of the requirements on compounds intended for use in agriculture and medicine. Because toxic compounds negatively influence metabolic processes in cells, many standard cytotoxicity assays are based on measurement of metabolisation rate of various artificial substrates.

- 15 Resulting product is then quantified e.g. by means of spectrometry. The assays can be easily modified for use in 96-well plates. For evaluation of cytotoxic effect of compounds of this invention, a microtiter assay based on quantification of metabolisation of Calcein AM was used. The assay is widely used in drug screening programs and in chemosensitivity testing. In live cells, Calcein AM is enzymatically hydrolysed and accumulation of resulting calcein is manifested by
20 green fluorescence.

- The cytotoxicity of the studied compounds was determined with human fibroblast lines MRC-5 (lung fibroblasts) and BJ (foreskin fibroblasts). The cells, cultured in DMEM (supplemented with 10% fetal calf serum, 4 mM glutamine, 100 IU/mL penicillin, 100 μ g/mL streptomycin) in a humidified CO₂ incubator at 37 °C, were redistributed into 96-well microtiter plates at appropriate
25 densities for their respective cell sizes and growth rates. After preincubation, test compounds in 3-fold dilutions were added in triplicates. Treatment lasted for 72 h and then calcein AM solution was added. The fluorescence of the live cells was measured at 485 nm/538 nm (excitation/emission)

with a Fluoroskan Ascent microplate reader (Labsystems). IC₅₀ values, the drug concentrations reducing number of viable cells to 50%, were determined from the dose-response curves.

The data obtained from a calcein AM viability/cytotoxicity assay are presented in Fig 11. The obtained IC₅₀ values show that compound **12** did not reach IC₅₀ value even in the concentration
5 100 µM with both tested primary cell lines.

Example 25

Genotoxicity of compound 12 in Ames test

Compound **12** was tested in classical Ames test to assess the mutagenic potential of the compound.

10 A positive test indicates that the chemical might act as a carcinogen (although a number of false-positives and false-negatives are known; Charnley G (2002).

The compound **12** was tested using Ames MPF™ 98/100 Mutagenicity Assay kit (Aniara, OH) that is employing *Salmonella typhimurium* strains TA100 (for the detection of base substitution mutations) and TA98 (for the detection of frameshift mutations) according to the instructions for
15 use.

The results summarized in Fig. 12 show that the compound **12** did not score positively in the assay up to the highest concentration tested (100 µM).

Example 26

Effect of compound 12 on induction of DNA breakage

Maintenance of genome integrity is essential for homeostasis and survival as impaired DNA damage response may predispose to grave pathologies such as neurodegenerative and immunodeficiency syndromes, cancer and premature aging (Mistrik et al., 2009). The potential effect of compound **12** on stimulation of DNA breakage was thus further estimated through
25 analysis of phosphorylated histone H2AX (gamma-H2AX) quantified by optimized automated single-cell image analysis (Mistrik et al., 2009). The compound **12** was tested in concentration of 100 µM, the highest concentration accessible due to the compound solubility. Induction of DNA breakage was tested using human U2OS cells. The U2OS cells were cultivated at standard cultivation condition (37°C, 5% CO₂). As a positive control, cells were irradiated by roentgen
30 radiation (Baltobloc) (0.5 Gy – 2 Gy), one hour later fixed and stained for γ-H2AX, a well established marker of the DNA damage. Parallel samples were treated by compound **12** for 24 hours, fixed and also stained for γ-H2AX. Microscopy analysis followed by computer based signal quantification was performed as described previously (Mistrik et al., 2009).

As shown in Fig. 13 compound **12** showed no effect on DNA damage confirming the zero
35 mutagenic effects observed in the Ames test.

Example 27

Preparations

The growth regulatory preparations usually contain from 0.1 to 99 % (w/w), preferably 0.1 to 95 % (w/w), of active ingredient mixture comprising a compound of formula **I**, from 1 to 99.9% (w/w) of a solid or liquid adjuvant, and from 0.1 to 25 % (w/w) of a surfactant. Whereas commercial products are usually formulated as concentrates, the end user will normally employ dilute formulations. The preparations may also comprise further ingredients, such as stabilisers, e.g. vegetable oils or epoxidised vegetable oils (epoxidised coconut oil 0;1, rapeseed *oil* or soybean oil), antifoams, e.g. silicone oil, preservatives, viscosity regulators, binders, tackifiers, and also fertilisers or other active ingredients. Preferred preparations have especially the following compositions: (% = percent by weight)

<u>A1. Emulsifiable concentrates</u>		a)	b)	c)	d)
	active ingredient mixture	5%	10%	25%	50%
15	calcium dodecylbenzenesulfonate	6%	8%	6%	8%
	castor oil polyglycol ether (36 mol of ethylene oxide)	4%	-	4%	4%
	octylphenol polyglycol ether (7-8 mol of ethylene oxide)	-	4 %	-	2 %
20	cyclohexanone	-	-	10 %	20 %
	arom. hydrocarbon mixture C ₉ -C ₁₂	85 %	78 %	55 %	16 %

Emulsions of any desired concentration can be obtained from such concentrates by dilution with water.

25

<u>A2. Solutions</u>		a)	b)	c)	d)
	active ingredient mixture	5 %	10 %	50%	90%
	1-methoxy-3-(3-methoxy-propoxy)-propane	-	20 %	20%	-
30	polyethylene glycol MW 400	20%	10 %	-	-
	N-methyl-2-pyrrolidone	-	-	30%	10%
	arom. hydrocarbon mixture C ₉ -C ₁₂	75 %	60 %	-	-

The solutions are suitable for use in the form of microdrops.

35

<u>A3. Wettable powders</u>		a)	b)	c)	d)
	active ingredient mixture	5 %	25 %	50 %	80 %

	sodium lignosulfonate	4 %	-	3 %	-
	sodium lauryl sulfate	2 %	3 %	-	4 %
	sodium diisobutylphenylene- sulfonate	-	6 %	5 %	6 %
5	octylphenol polyglycol ether (7-8 mol of ethylene oxide)	-	1 %	2 %	-
	highly dispersed silicic acid	1 %	3 %	5 %	10 %
	kaolin	88 %	62 %	35 %	-

The active ingredient is mixed thoroughly with the adjuvants and the mixture is thoroughly ground
 10 in a suitable mill, affording wettable powders which can be diluted with water to give suspensions
 of any desired concentration.

	<u>A4. Coated granules</u>	a)	b)	c)
	active ingredient mixture	0.1 %	5%	15%
15	highly dispersed silicic acid	0.9 %	2 %	2 %
	inorganic carrier (0.1 -1 mm) e.g. CaCO ₃ or SiO ₂	99.0 %	93 %	83 %

The active ingredient is dissolved in methylene chloride and applied to the carrier by spraying, and
 20 the solvent is then evaporated off in vacuo.

	<u>A5. Coated granules</u>	a)	b)	c)
	active ingredient mixture	0.1 %	5 %	15 %
	polyethylene glycol MW 200	1.0 %	2 %	3 %
25	highly dispersed silicic acid	0.9 %	1 %	2 %
	inorganic carrier (AE 0.1 -1 mm) e.g. CaCO ₃ or SiO ₂	98.0 %	92 %	80 %

The finely ground active ingredient is uniformly applied to the carrier moistened with polyethylene
 30 glycol. Non-dusty coated granules are obtained in this manner.

	<u>A6. Extruder granules</u>	a)	b)	c)	d)
	active ingredient mixture	0.1 %	3 %	5 %	15 %
	sodium lignosulfonate	1.5 %	2 %	3 %	4 %
35	carboxymethylcellulose	1.4 %	2 %	2 %	2 %
	kaolin	97.0 %	93 %	90 %	79 %

The active ingredient is mixed and ground with the adjuvants, and the mixture is moistened with
 water. The mixture is extruded and then dried in a stream of air.

<u>A7. Dusts</u>		a)	b)	c)
	active ingredient mixture	0.1 %	1 %	5 %
	talc	39.9 %	49 %	35 %
5	kaolin	60.0 %	50 %	60 %

Ready-to-use dusts are obtained by mixing the active ingredient with the carriers and grinding the mixture in a suitable mill.

<u>A8. Suspension concentrates</u>		a)	b)	c)	d)
10	active ingredient mixture	3%	10%	25%	50%
	ethylene glycol	5 %	5 %	5 %	5%
	nonylphenol polyglycol ether (15 mol of ethylene oxide)	-	1 %	2 %	-
	sodium lignosulfonate	3 %	3 %	4 %	5 %
15	carboxymethylcellulose	1 %	1 %	1 %	1%
	37 % aqueous formaldehyde solution	0.2 %	0.2 %	0.2 %	0.2%
	silicone oil emulsion	0.8 %	0.8 %	0.8 %	0.8%
	water	87 %	79 %	62 %	38%
20					

The finely ground active ingredient is intimately mixed with the adjuvants, giving a suspension concentrate from which suspensions of any desired concentration can be obtained by dilution with water .

25 Example 28

Gel formulation

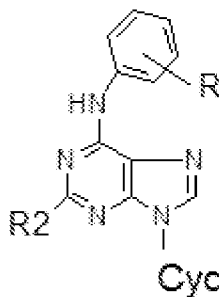
The names of the formulation components are given according to the terminology of the registering authorities and their quantity is in grams per 100 g.

30	Gel	/100 g
	Active compound 2-chloro-6-(4-hydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine	1.0 g
	butylhydroxytoluenum (Nipanox BHT)	0.2 g
	butylparaben (Nipabutyl)	0.2 g
35	diethylene glycol monoethyl ether (Transcutol P)	10.0 g
	silica colloidalis anhydrica (Zeopharm 177)	5.0 g
	propylene glycol laurate (Lauroglycol FCC)	83.6 g

The gel consistence may be additionally modified by addition of silica colloidalis anhydrica. It is again expected that the transdermal Transcutol P/Lauroglycol FCC system will increase the efficiency of active compound. Silica colloidalis anhydrica will probably slow down the penetration of the active substance.

CLAIMS

1. Substituted-6-anilino-9-heterocyclylpurines of general formula **I**



(I)

- 5 and the pharmaceutically acceptable salts thereof with alkali metals, ammonium or amines, in the form of racemates or optically active isomers, as well as their addition salts with acids, wherein **R** denotes one to five substituents independently selected from the group comprising hydrogen, halogen, hydroxy, amino, alkyloxy and alkyl group, **R2** is selected from the group comprising amino, halogen, hydroxy, thio, and alkylthio group, **Cyc** is five- or six-membered heterocyclic ring containing one oxygen atom.
2. Substituted-6-anilino-9-heterocyclylpurines of general formula **I** according to claim 1, wherein **Cyc** is selected from tetrahydropyranyl and tetrahydrofuranyl, preferably from tetrahydropyran-2-yl and tetrahydrofuran-2-yl.
3. The substituted-6-anilino-9-heterocyclylpurines of general formula **I** according to claim 1 or 2, selected from the group consisting of 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-anilino-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, nitro, thio, methylthio, methyl)-6-(3-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(4-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-fluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-fluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(4-fluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-bromoanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, nitro, thio, methylthio, methyl)-6-(3-bromoanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro,

fluoro, bromo, iodo, amino, nitro, thio, methylthio, methyl)-6-(4-bromoanilino)-9-
(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, nitro,
thio, methylthio, methyl)-6-(2-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-
yl)purine, 2-(chloro, fluoro, bromo, idodo, amino, nitro, thio, methylthio, methyl)-6-(3-
5 methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo,
iodo, amino, hydroxy, thio, methylthio)-6-(4-methoxyanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
(2-ethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo,
iodo, amino, hydroxy, thio, methylthio)-6-(3-ethoxyanilino)-9-(tetrahydropyran-2-yl or
10 tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
(4-ethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-aminoanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo,amino, hydroxy, thio, methylthio)-6-(3-
aminoanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo,
15 iodo, amino, hydroxy, thio, methylthio)-6-(4-aminoanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo,amino, hydroxy, thio, methylthio)-6-(2-
methylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo,
iodo,amino, hydroxy, thio, methylthio)-6-(3-methylanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo,amino, hydroxy, thio, methylthio)-6-(4-
20 methylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo,
iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxyanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
(3-hydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
bromo, iodo, amino, hydroxy, thio, methylthio)-6-(4-hydroxyanilino)-9-(tetrahydropyran-2-yl or
25 tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
(2,3-difluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,4-difluoroanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
(2,3,4-trifluoroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
30 bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,4,5-trifluoroonilino)-9-(tetrahydropyran-2-yl
or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo,amino, hydroxy, thio, methylthio)-6-
(2,3-dichloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
bromo, iodo,amino, hydroxy, thio, methylthio)-6-(2,4-dichloroanilino)-9-(tetrahydropyran-2-yl or
tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-
35 (2,4-dimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro,
bromo, iodo, amino, nitro, thio, methylthio, methyl)-6-(2,3-dimethoxyanilino)-9-(tetrahydropyran-

2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3,4-dimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,5-dimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3,4,5-trimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(amino, chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,4,6-trimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, amino, nitro, thio, methylthio, methyl)-6-(2,3-dimethylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,4-dimethylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3,4-dimethylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3,5-dimethylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,3-dihydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,4-dihydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2,5-dihydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(amino, chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3,5-dihydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-hydroxy-2-methylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-hydroxy-4-methylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxy-5-methylanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-hydroxy-2-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(3-hydroxy-4-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxy-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-hydroxy-5-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(4-hydroxy-3,5-dimethoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloro-5-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-chloro-3-methoxyanilino)-9-(tetrahydropyran-2-yl or

tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-bromo-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-methoxy-3-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo, iodo, amino, hydroxy, thio, methylthio)-6-(2-methoxy-4-chloroanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine,

and the pharmaceutically acceptable salts thereof with alkali metals, ammonium or amines, in the forms of racemates or optically active isomers, as well as their addition salts with acids.

4. The substituted 6-anilino-9-heterocyclylpurines of general formula **I** according to claim 1 or 2, selected from the group consisting of 2-(chloro, fluoro, bromo)-6-(3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-hydroxy-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-chloro-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(2-bromo-3-methoxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine, 2-(chloro, fluoro, bromo)-6-(4-hydroxyanilino)-9-(tetrahydropyran-2-yl or tetrahydrofuran-2-yl)purine,

and the salts thereof with alkali metals, ammonium or amines, in the forms of racemates or optically active isomers, as well as their addition salts with acids.

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5. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 for inhibition of stress in whole plants, in plant organs, in plant tissues and/or in plant cells.

6. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 for stimulation of growth and development of plant shoots without inhibiting the development of plant roots.

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7. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 for cloning of plants, plant organs, plant tissues and/or plant cells.

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8. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 as plant antistress factors, in particular for increasing the yield and quality of agricultural products in harmful conditions such as drought, heat, salinity, light, freezing and flowage.

9. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 for regulation of plant stress, plant senescence, plant growth and/or plant development.

35

10. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 as plant antistress factors in agriculture to dispatch grain filling, to increase grain and fruit size of plants, to shorten plant seed germination period, to increase the yield and quality of agricultural products, to enhance root and shoot growth, and/or to increase stress resistance to environmental factors.

11. Use of substituted 6-anilino-9-heterocyclylpurines according to any one of claims 1 to 4 for inhibiting stress-induced senescence in the production of crops, in particular cereals, beet, pomes, drupes and soft fruits, leguminous plants, oil plants, cucumber plants, citrus fruit, vegetables, tobacco, nuts, eggplants, sugar cane, tea, vine gpapes, hops, bananas, natural rubber and medicinal plants, and/or ornamentals.

12. Preparations for inhibition of plant stress, characterized in that they contain at least one substituted 6-anilino-9-heterocyclylpurine of general formula I according to any one of claims 1 to 4 or a salt thereof with metals, ammonium or amines, or an addition salt thereof with acids, and at least one auxiliary substance.

13. A method of inhibiting plant stress, characterized in that at least one substituted 6-anilino-9-heterocyclylpurine of general formula I according to any one of claims 1 to 4 or a salt thereof with metals, ammonium or amines, or an addition salt thereof with acids, is applied on whole plant, plant organ, plant tissue and/or plant cell.

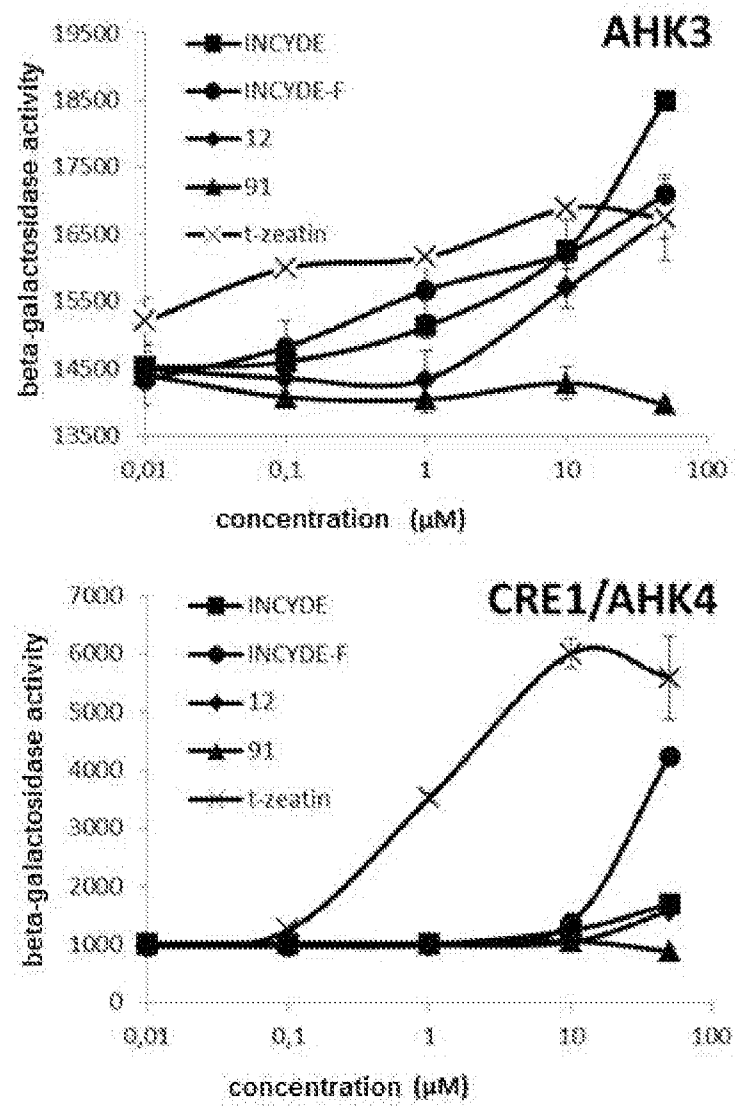


FIGURE 1

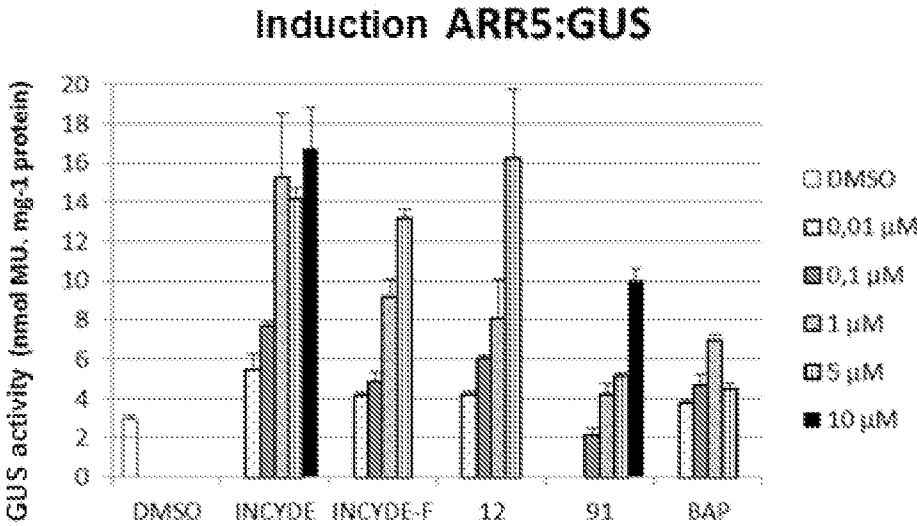


FIGURE 2

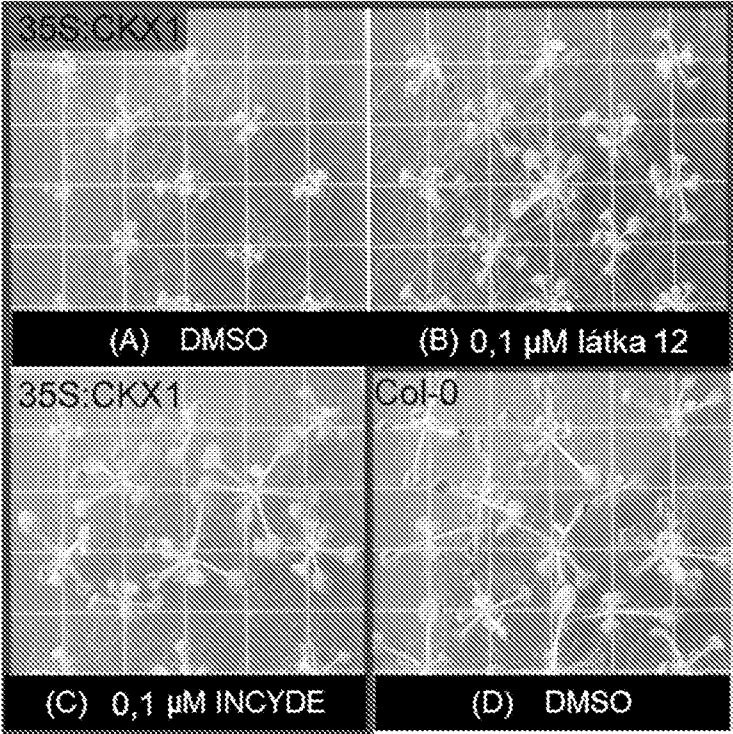


FIGURE 3

Effect on shoot of CKX1 overexpressing
Arabidopsis seedlings

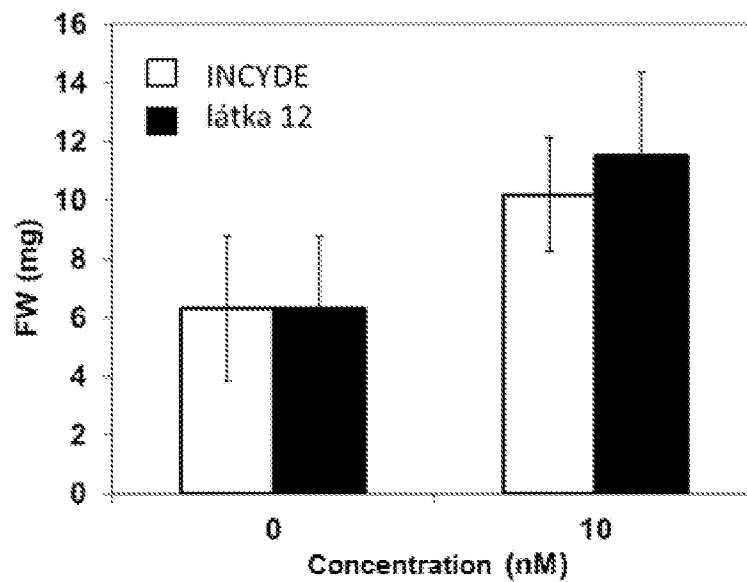


FIGURE 4

Effect on root system of CKX1 overexpressing
Arabidopsis seedlings

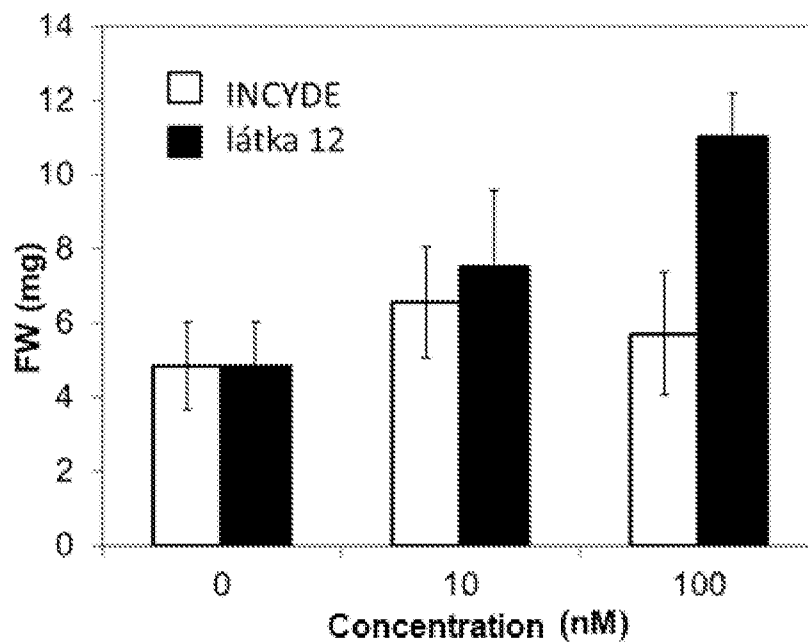
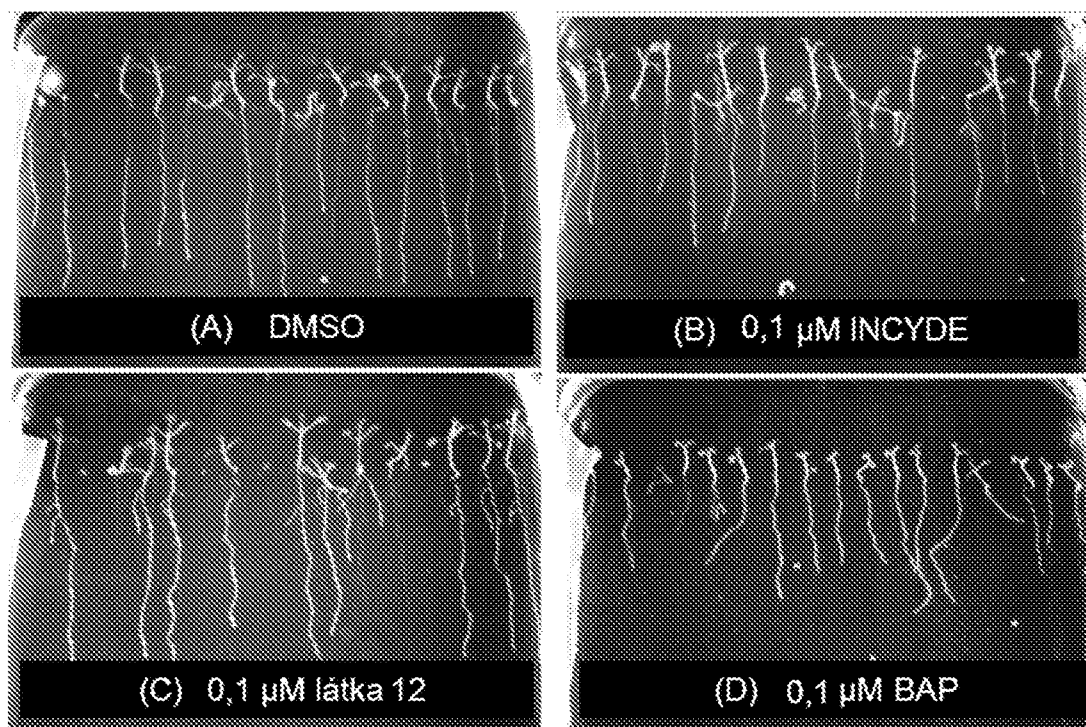


FIGURE 5

**FIGURE 6**

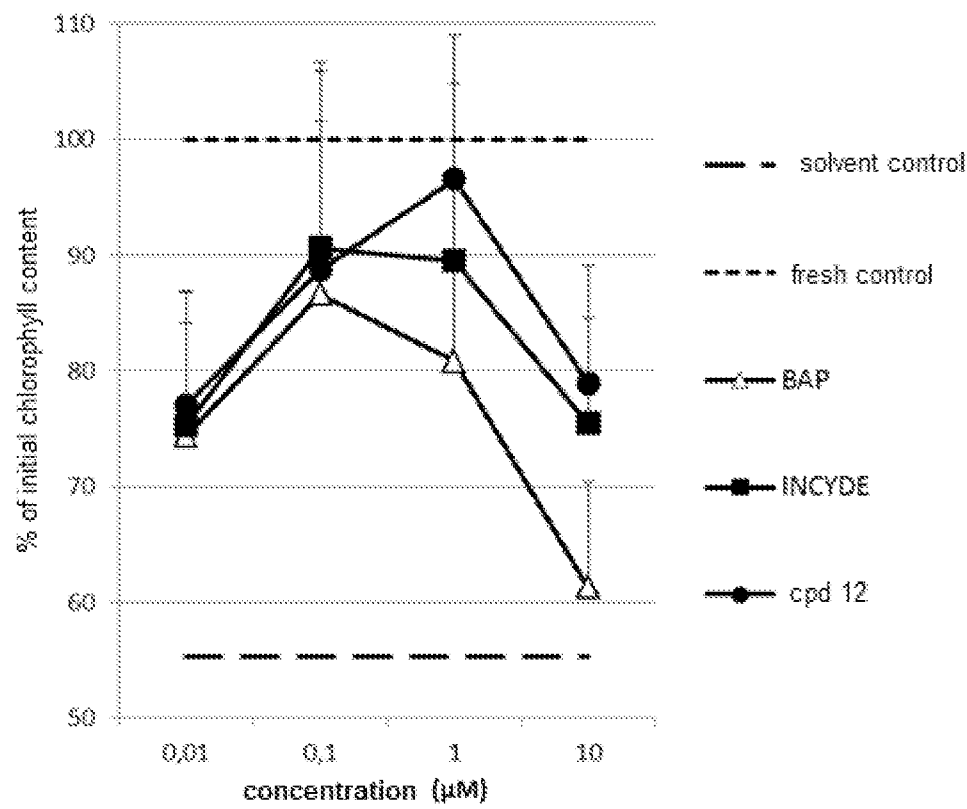


FIGURE 7



FIGURE 8

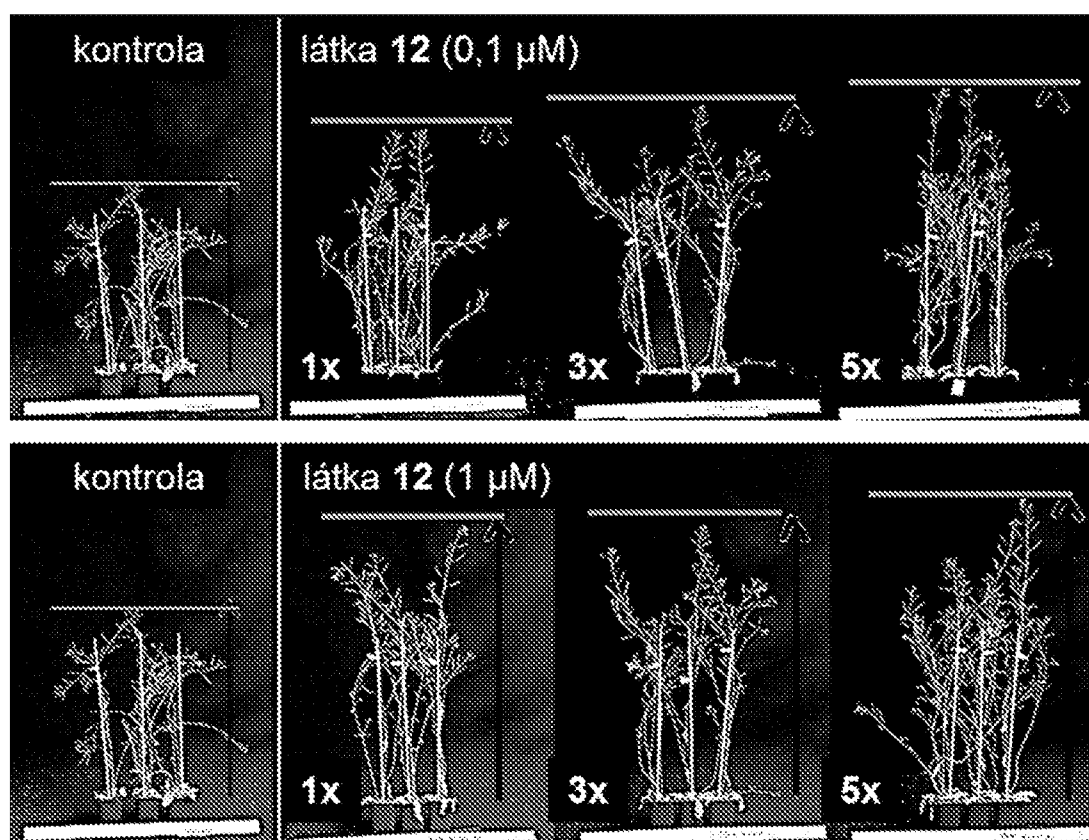


FIGURE 9

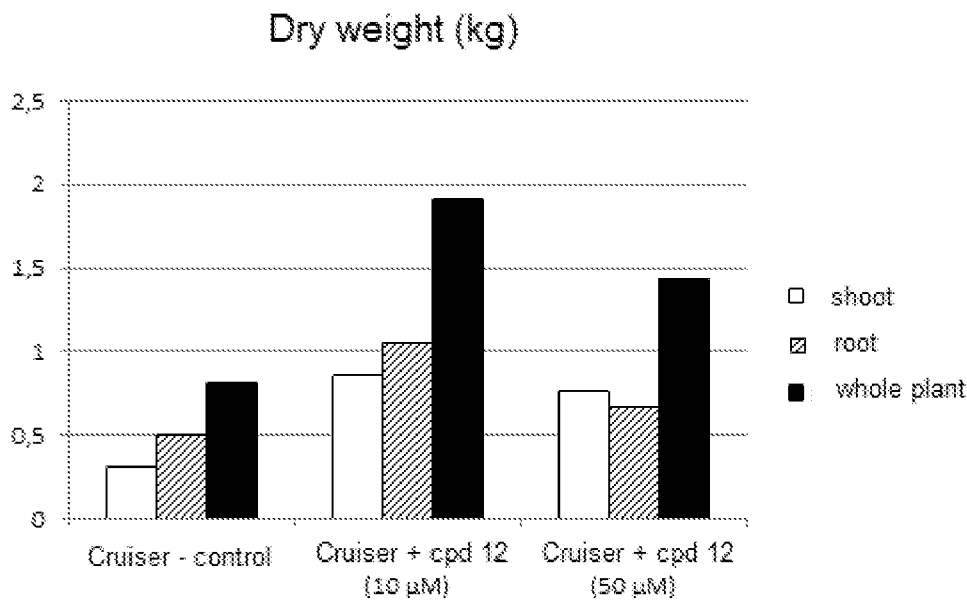


FIGURE 10

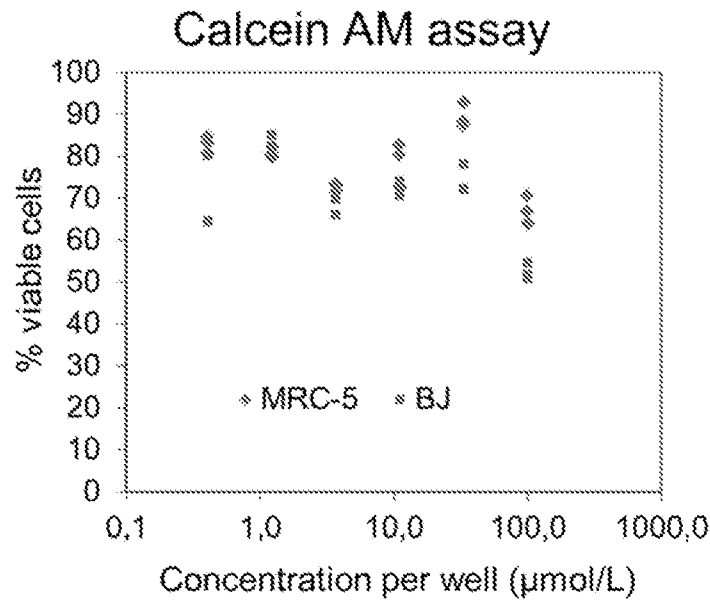


FIGURE 11

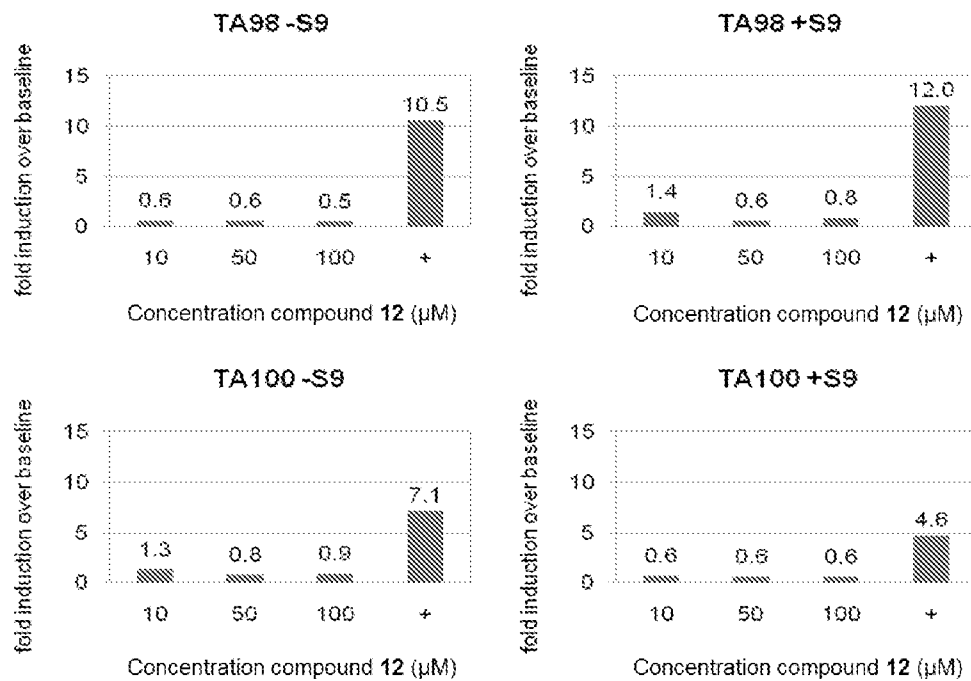


FIGURE 12

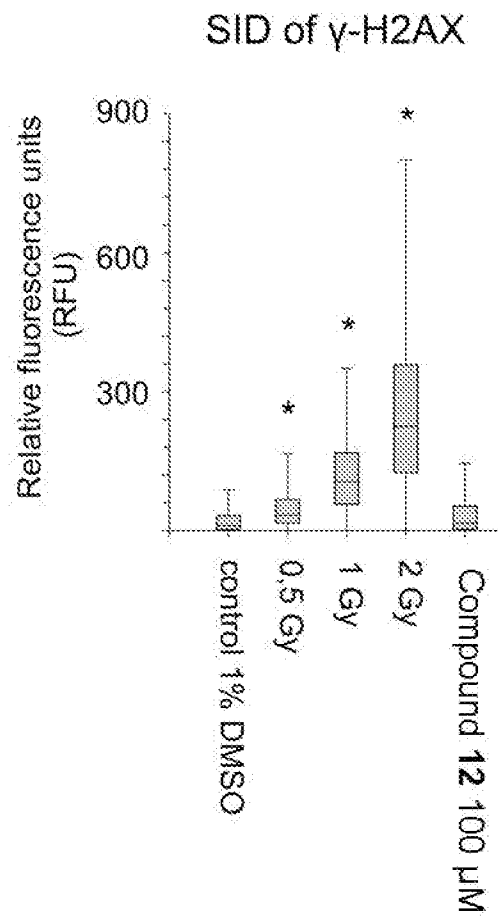


FIGURE 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2015/050014

A. CLASSIFICATION OF SUBJECT MATTER INV. C07D473/16 A01N43/713 A01N43/90 C07D473/34 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07D A01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009/003428 A2 (UNIVERZITA PALACKEHO V OLOMOUC [CZ]; UNIV BERLIN FREIE [DE]; SPICHAL L) 8 January 2009 (2009-01-08) abstract; claims 1-12	1-13
A	WO 2010/130233 A1 (UNIVERZITA PALACKEHO V OLOMOUC [CZ]; BIOPATTERNS S R O [CZ]; SZUCOVA L) 18 November 2010 (2010-11-18) abstract; claims 1-9	1-13
	----- -/-	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
18 February 2016		26/02/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Goss, Ilaria

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2015/050014

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International application No

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