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Review

DESIGN, SYNTHESIS AND PROPERTIES OF SYNTHETIC CYTOKININS. RECENT ADVANCES ON THEIR APPLICATION

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Summary: The influence of exogenously applied compounds on the growth and morphogenesis of plant tissues as well as on the growth and development of plants have been studied for more than 6 decades. Nevertheless, the discovery of new bioactive compounds having selective activity and possessing specific applicability is a very difficult and sustained process. The present review presents design, purposeful synthesis, activity and possibilities for application of novel biologically active compounds, focusing on these with cytokinin-like properties. Compounds to be discussed include mainly the phenylureas, which represent the largest group of the non-purine-type cytokinins. The phenylureas exhibit also other activities, such as growth-regulating, antiphytoviral, antimutagenic, pesticidal, etc.. Many synthetic growth regulators may be important and useful for plant applications due to their high activities and economical advantages - considerably less expensive and toxic. In addition, these synthetic compounds are metabolically stable, resistant to oxidases, and biologically active at low concentrations. Present and future possibilities regarding the practical application of newly developed bioactive compounds with cytokinin-like properties in plant kingdom are discussed as well.

Keywords: application; biological activity; cytokinin; design; plant growth regulator; purposeful synthesis.

Abbreviations: 3-CP-2PU – 1-(3-chlorophenyl)-3-(2-pyridyl)urea; 3-CP-4MPU – 1-(3-chlorophenyl)-3-(4-methyl-2-pyridyl)urea; 4PU-30 – 1-phenyl-3-(2-chloro-4-pyridyl)urea; Rubisco – ribulose bisphosphate carboxylase/oxygenase (EC 4.1.1.39); PEPC – phospho*enol*pyruvate carboxylase (EC 4.1.1.31); SPGRs – synthetic plant growth regulators; VOCs – volatile organic compounds.

Strategy to design and synthesis of new biologically active compounds

The influence of exogenously applied compounds on the growth and morphogenesis of plant tissues as well as on the growth and development of plants have been studied for more than 6 decades. The manipulation of plant cells, tissues, and organs in culture, with important applications in propagation and genetic modification of plants, is highly dependent on the use of appropriate growth regulator

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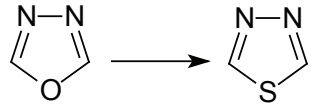
regimes. Since the discovery of auxin/cytokinin responses in plant cell culture systems, many additional compounds have been discovered that influence culture growth and morphogenesis. Nevertheless, the impact these compounds have produced on the successful development of regeneration systems through empirical testing has been substantial. It is known that the discovery of new biologically active compounds having selective activity and possessing specific applicability is a very difficult and sustained process.

The general strategy to design new bioactive compounds includes three basic directions: 1) structural modification of bioactive natural products. Thus, active natural substances can be used to obtain different substituted analogues, sometimes with enhanced activity compared to those of the parent compounds; 2) the available information about the quantitative analysis of chemical structure - biological activity relationships (QSAR) of different series of compounds is also a good basis as a starting point for the synthesis of new compounds and the activity optimization. Despite significant experimental data, a theory associating the structural elements with the appearance of a definite type of physiological activity has not been formulated yet; 3) bioisosteric transformations that are one of the most successful techniques of bioactive compounds design (Lipinski, 1986). Some examples of groups that are bioisosterically equivalent include phenyl with thiophenyl, and carboxylic acid with tetrazole. Identifying bioisosteric analogues of more complex groups, however, is not so trivial. The replacement of oxygen for sulfur in the heterocyclic ring represents an example of an approach that is commonly known as bioisosterism. *Example:* Bioisosteric analogue of 1,3,4-oxadiazole (O) is 1,3,4-thiadiazole ring (S).

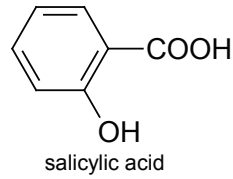


The designed "molecular model" of a supposed bioactive compound(s) is realized in the way of the purposeful synthesis. This approach allows the combination of different functional groups from active biomolecules proved in a single structure resulting in new compounds with improved activity. This kind of work leads to a series of potentially active synthetic compounds, chemically related to the natural substances.

The synthetic compounds obtained which involve important deviations from the structure of initial compounds have some advantages: a) they are metabolically stable because the chemical modifications turn them into less susceptible to degradative enzymes (oxidases, etc.); b) they manifest a large scale of physiological activities; c) they are biologically active at lower concentrations; d) they have an enhanced duration of biological action; e) they are considerably less expensive and toxic.

Some examples of designation and obtaining new biologically active compounds discussed below merely illustrate the potential of this biomimetic approach for preparing analogues with novel and useful biological activities; future researchers toward the evolution of new compounds' activity will greatly expand the arsenal of growth-regulating activity and make significant contributions to both fundamental science as well as practical technologies.

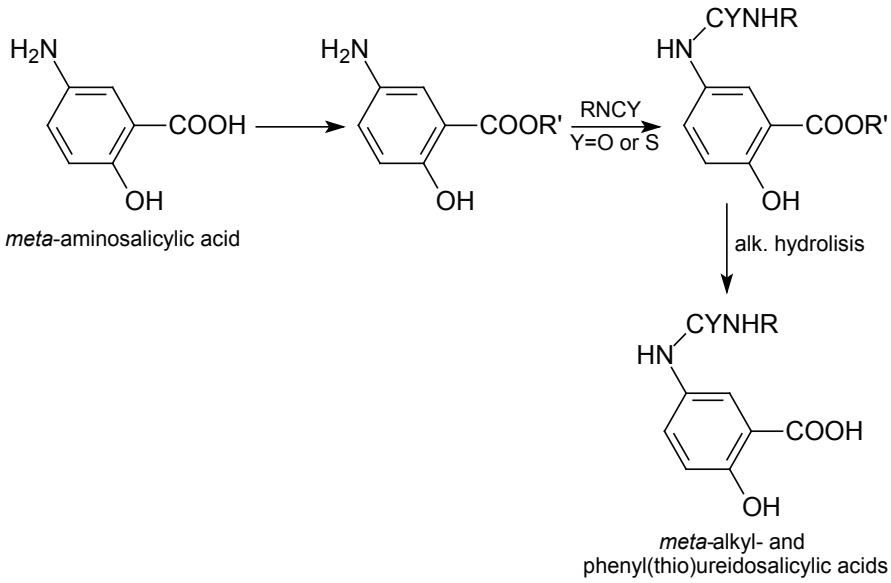
Bioactive natural substance – salicylic acid



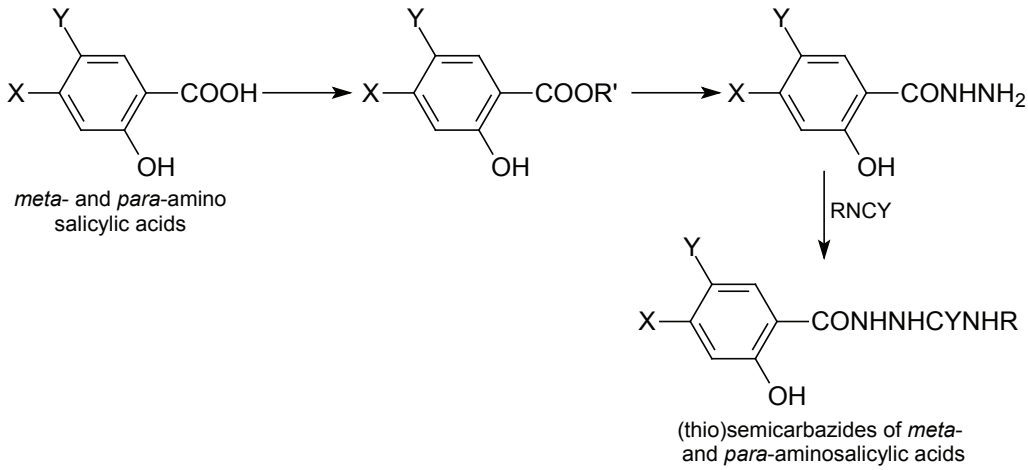
salicylic acid

Synthesis of a new series of active analogues – derivatives of meta- and para-aminosalicylic acids (Vassilev and Yonova, 1976; Yonova and Vassilev, 1983; Yonova and Vassilev, 1985).

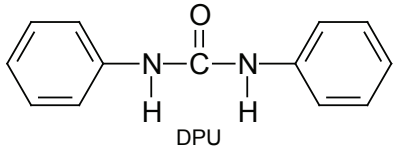
I.



II.



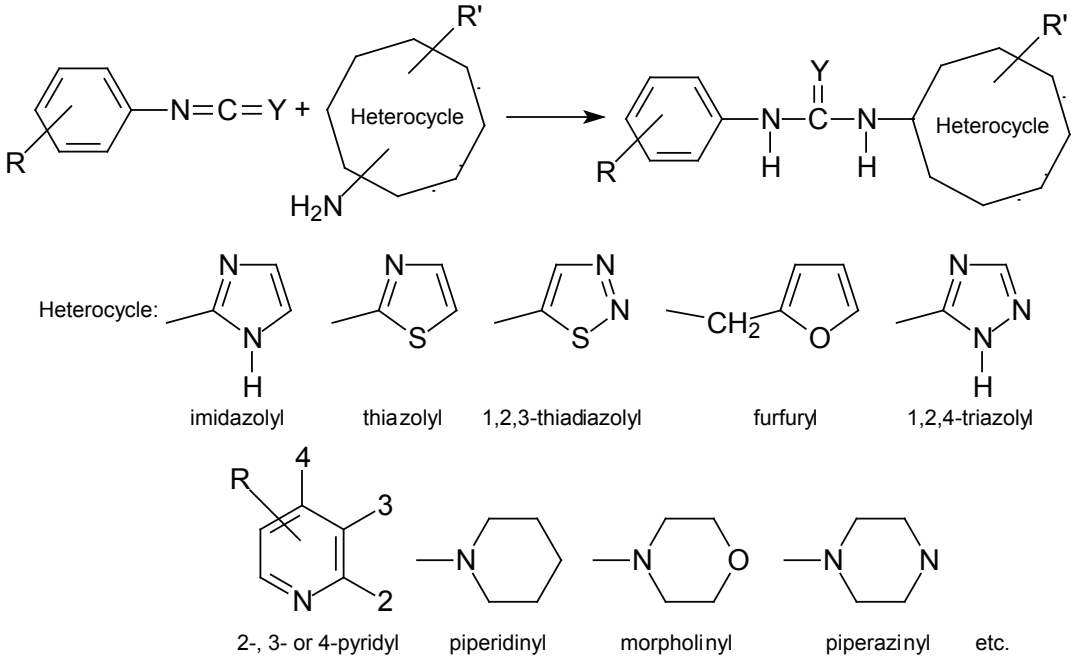
Bioactive natural substance – 1,3-diphenylurea



Synthesis of a new series of active analogues – phenyl(thio)urea derivatives, containing 5- or 6-member heterocyclic rings.

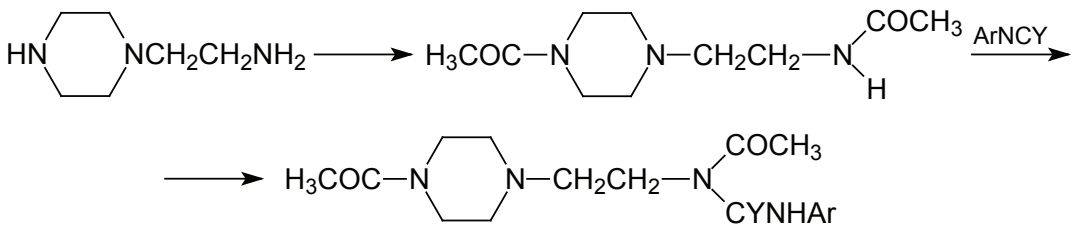
I. Synthesis of phenyl–NHCYNH-heterocycle (imidazolyl, thiazolyl, thiadiazolyl, triazolyl, furfuryl, pyridyl, NH-heterocycles - piperidyl, morpholinyl, piperazinyl, etc.), these compounds combine in single structure the properties of two phytophores (heterocycle and (thio)urea bridge).

The physiological activity of these phenyl-heterocyclic(thio)urea derivatives is very sensitive to the type of heterocycle (Yonova and Vassilev, 1987, 1992; Yadav et al., 1994; Yonova and Stoilkova, 2005a, b).



II. Synthesis of 1-[2-aryl(thio)carbamoylaminoethyl]-4-acetyl-piperazines, these compounds contain three active structural units: piperazine ring, ethylene and aryl(thio)carbamoyl groups (Kempter et al., 1983; Toncheva and Yonova, 2002).

To determine the biological activity type of newly synthesized compounds a screening strategy including diverse selective and specific bioassay systems is used and their differential responses to the different compounds are investigated.



Biologically active compounds possessing cytokinin-like properties

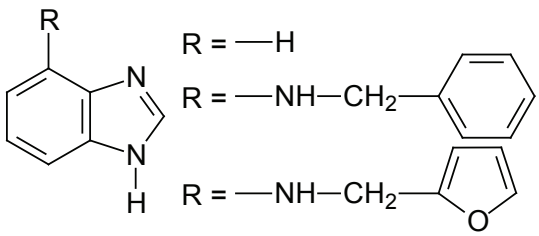
The present review is a sharply focused summary and assessment of the relevant literature and our own results concerning preparation, activity and possibility for application of novel compounds exhibiting cytokinin-like activity.

It is well known that two chemically different groups of substances possess cytokinin effects. They are purine (*purine cytokinins*) and non-purine (*non-purine cytokinins*) compounds. The structural variations in ring systems as well as in the side-chain can markedly influence the biological activity of both types of cytokinins.

Non-purine cytokinins

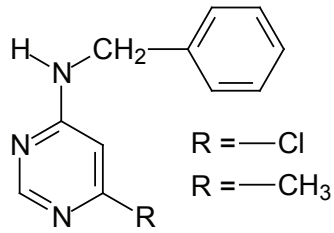
The cytokinin activity of some groups of chemical substances possessing either separate structural elements of the adenine cytokinins or a structute other than that of the adenine cytokinins has been already reported. Here, we show certain key representatives of subgroups of the non-purine cytokinins.

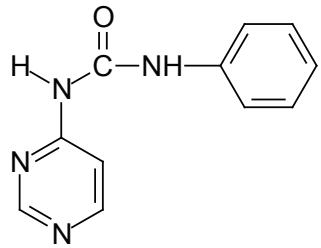
Benzimidazoles (contain the imidazole ring of the adenine cytokinins).



Benzimidazole (R=H), 4-benzyl-aminobenzimidazole and 4-furfurylamino-benzimidazole (synthetic compounds) demonstrate cytokinin activity at concentrations of 20-40 μM on tobacco callus growth and chlorophyll retention (Kulaeva, 1973). The cytokinin activities of two series of 4,5-disubstituted imidazoles are shown using a wheat leaf chlorophyll retention bioassay (Cavender et al., 1988).

Pyrimidines (contain the pyrimidine ring of the adenine cytokinins).

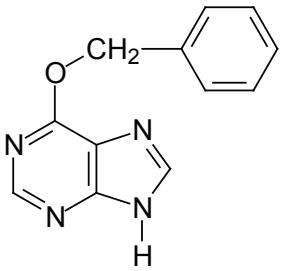


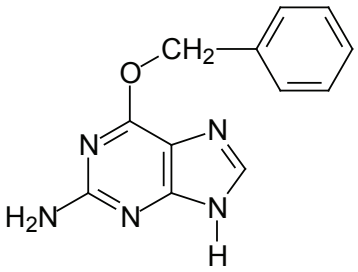


4-Benzylaminopyrimidine (R=H) is inactive, but cytokinin activity is observed in pyrimidine's derivatives: 4-benzylamino-6-methyl-pyrimidine ($R=CH_3$) exhibits cytokinin activity similar to that of benzylaminopurine (BAP, 0.01 μM) but at a 100-times higher concentration, 4-(3'-phenylureido) pyrimidine possesses cytokinin activity in the tobacco callus bioassay at 4.7 μM, while their 6-chloro derivatives 4-benzylamino-6-chloro-pyrimidine (R=Cl) are significantly active cytokinins (Takahashi et al., 1978).

O-6-substituted derivatives of hypoxanthine and guanine (other two bases constructing ribonucleic acids and containing purine ring).

Most attention has been paid to substitution of nitrogen for oxygen between the purine ring (C^6-position) and the side chain. The established

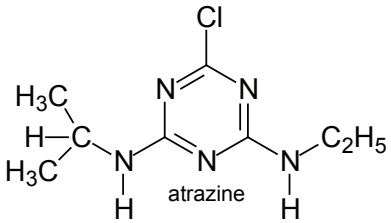


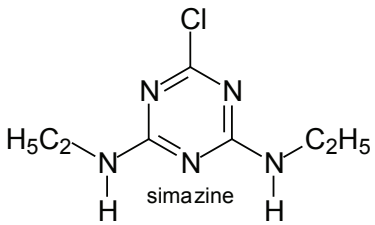


cytokinin activity of synthetically obtained O-6-substituted derivatives of hypoxantine and guanine shows that an intact purine ring is necessary for high cytokinin activity. Among them, O-6-benzylhypoxanthine and O-6-benzylguanine display rather cytokinin activity near that of kinetin (Hashizume et al., 1976).

Hormonal herbicides

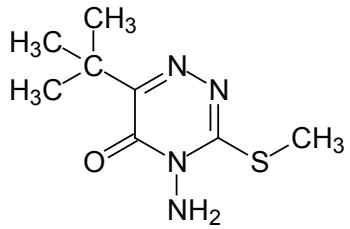
Simmetric-triazine herbicides (atrazine, simazine, prometrine).





The biological properties of these compounds include also cytokinin activity - they give better callus yield in sorghum and retain chlorophyll degradation at optimum concentrations less than 10^{-7} M (Iliev, 1973; Nadar et al., 1975).

Asimmetric-triazinone herbicides (metribuzin) - a herbicide with cytokinin activity

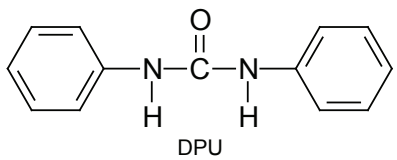


Metribuzin and BAP exerted similar effects on the structural and functional characteristics of the photosynthetic apparatus of agricultural plants (wheat, sugar beet) (Chernyad'ev, 2000).

Aromatic ureas - the most important group of non-purine cytokinins

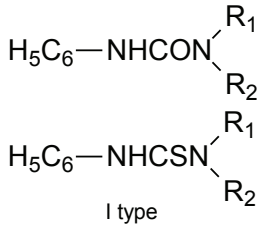
1,3-Diphenylurea and related derivatives

DPU is isolated from coconut milk and found to possess growth-promoting properties (Shantz and Steward, 1955).

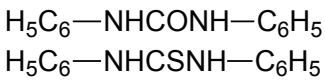


In 1960 – 1968, Bruce, Zwar and Kefford synthesized and tested for the first time more than 500 derivatives of 1-phenyl- (I type) and 1,3- diphenyl- (II type) ureas and thioureas for their cytokinin

activity in various bioassay systems used also by the adenine cytokinins.



I type



II type

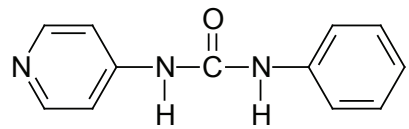
The authors found some compounds, including DPU, to be consistent with or more active than kinetin. *Structure – activity relationships* for the above mentioned group of compounds are established: a) a purine ring is not an absolute requirement for cytokinin activity; b) an intact urea –NH–CO–NH- bridge with a phenyl ring attached (I type) is required to exhibit activity. Substitution on the ring with phenyl and electronegative groups increases the activity in the order: *meta* > *para* > *ortho.* c) In general, the compounds of the II type are more active than those of type I; d) among compounds of II type, compounds with one unsubstituted phenyl ring display the highest activity; e) an urea NHCONH bridge confers higher activity than a thiourea NHCSNH linkage. f) substitution of a benzyl group for the phenyl group of DPU and some other related derivatives converts them from active cytokinins to antagonists [a difference disparity of adenine cytokinins] (Bruce et al., 1965; Kefford et al., 1966; 1968).

At the same time, Bulgarian researchers synthesized and investigated a significant number of urea and thiourea compounds for their cytokinin activity (Karanov et al., 1969; Karanov and Vassilev, 1970).

Despite the high cytokinin activity, the phenyl(thio)ureas have not received much attention, they even have often been neglected when considering cytokinins for a long time.

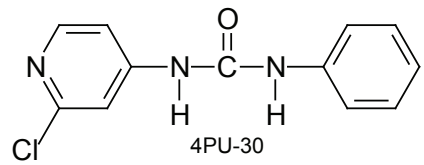
1-Phenyl-3-(4-pyridyl)urea and related derivatives

A matter of ten years later (1976 – 1981), Japanese researchers synthesized and examined as cytokinins more than 150 kinds of 1-phenyl-3-(4-pyridyl)urea. The last compound is obtained by Shudo (1974) as DPU analog and found to be highly active [4PU, 10^{-7}M] in tobacco bioassay. Its isomers, 1-phenyl-3-(3-pyridyl)urea (10^{-5}M) and 1-phenyl-3-(2-pyridyl)urea, were lower or almost inactive.

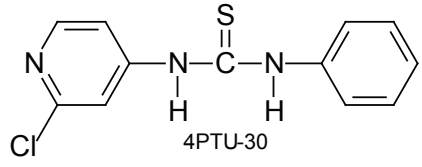


4PU

Isogai proposed that the introduction of Cl into position 2 of the pyridyl ring of 4PU may enhance the activity (like 4-benzylamino-6-chloro-pyrimidine). Really, the product 1-phenyl-3-(2-chloro-4-pyridyl)urea [4PU-30, 10^{-9}M] showed surprisingly high activity in tests done by Takahashi et al. (1978) which was about 10, 100 and 10 000 times higher than that



4PU-30

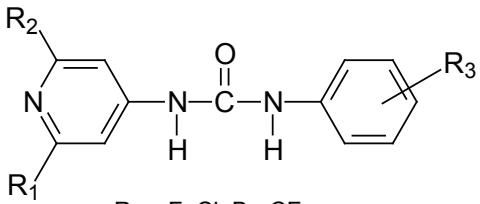


4PTU-30

of BAP, 4PU and DPU, respectively. The thiourea analog of 4PU-30 possesses also high cytokinin activity [4PTU-30, 10^{-9} M].

Later, Okamoto et al. (1981) found that the dichlorinated derivative of 4PU - 1-phenyl-3-(2,6-dichloro-4-pyridyl)urea was one of the most biologically active chemicals known so far [activity below 10^{-13} M].

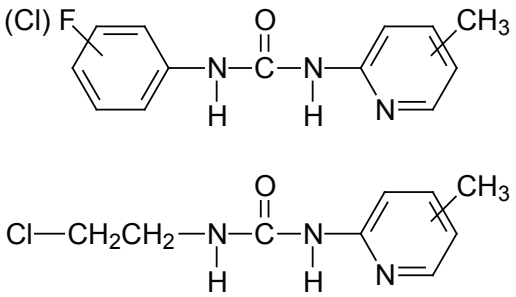
Structure – activity relationships of 1-phenyl-3-(pyridyl)urea derivatives are established:



R_1 = F, Cl, Br, CF_3
R_2 = H, Cl, Br, Me, OMe
R_3 = H, 3-F

a) mono- or di-substitution on the phenyl ring always results in a reduction of activity, with the exception of 3-F; b) the most favourable position for mono-substitution on the pyridyl ring is 2- , whereas for di-substitution there are two α-positions (2,6-); c) substitution of urea with a thiourea bridge between both rings seems to give similar activity; d) in general, the presence of an intact urea/ thiourea bridge connecting the phenyl ring to a 4-pyridyl ring is a necessary structural requirement for giving high cytokinin activity.

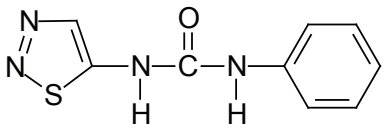
In addition to the series of 1-phenyl-3-(pyridyl)urea derivatives, there are also results on the cytokinin activity of 1-(fluoro- or chlorophenyl)-3-pyridylureas and of 1-(2-chloroethyl)-3-pyridylureas obtained in our laboratory (Vassilev et al., 1984; Yonova and Vassilev, 1987; Izvorska et al., 1991).



1-Phenyl-3-(heterocycle)ureas and related derivatives

1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea

Tthidiazuron (Dropp) – its cytokinin activity is detected in a *Phaseolus lunatus* L. callus bioassay (Mok et al., 1982), where it is more active than zeatin and almost as active as 4PU-30.



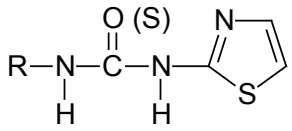
Thidiazuron

Structure – activity relationships of 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea derivatives are established: a) the replacement of the phenyl ring with other ring structures results in a reduction of activity in the following order: phenyl > 2-pyridyl > benzyl = furfuryl > thiadiazolyl ring; b) methylation of urea bridge [–N(CH_3)CONH–] results in a marked reduction in cytokinin activity. Therefore, all available analogues of thidiazuron are less active than the parent compound.

1-Phenyl-3-(2-thiazolyl or furfuryl)ureas/ thioureas

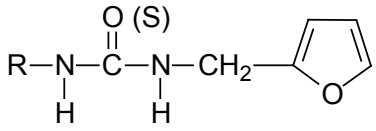
These compounds have manifested high activity in two cytokinin bioassays *(Amaranthus*-betacyanin synthesis and *Raphanus* cotyledon enlargement) and the following relationships have been found:

a) the thiazol ring provides significantly enhanced activity whereas the furan ring has little effect; b) the presence of unsubstituted or *meta*-substituted phenyl ring tends to exhibit high activity (Yonova and Stoilkova, 2005a).



R = phenyl or substituted phenyl

1-aryl and alkyl-3-(2-thiazolyl) ureas and thioureas

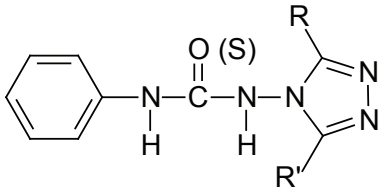


R = phenyl or substituted phenyl

1-aryl and alkyl-3-(2-furfuryl) ureas and thioureas

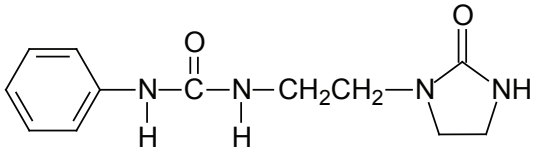
1-Phenyl-3-(1,2,4-triazolyl)ureas and thioureas

They are evaluated as cytokinins and antisenescence agents (Fawsi and Quabedeaux, 1976).



1-Phenyl-3-[2-(2-oxo-1-imidazolidinyl) ethyl]urea

EDU (ethylendiurea) - displayed cytokinin-like activity in the *Nicotiana tabacum* L. callus bioassay and much higher effectiveness in arresting senescence than kinetin (Lee and Chen, 1982).

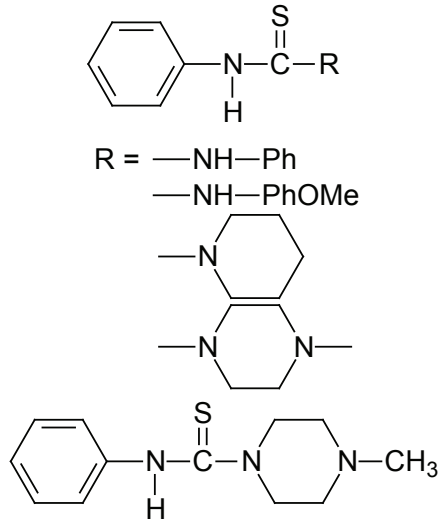


EDU

Some EDU analogues are synthesized and tested for protection effect against oxygen-derived free radical damage in mammals, by inducing endogenous antioxidant enzymes. Among them, the most active compound is a *para*-chlorophenyl derivative that increases SOD and CAT activities (by 51 and 95%, respectively) in human cells (Kerr and Boswell, US Patent, 1991).

1-Phenyl-3-(phenyl- or NH-heterocycles - piperidyl, piperazinyl)thioureas

Their antioxidant properties are proved for seven thiourea derivatives, including 1-methyl-4-phenylthiocarbamoyl-piperazine (Krivenko et al., 2000).



1-methyl-4-phenylthiocarbamoyl-piperazine

The last compound and its analogues have also been obtained in our laboratory (Toncheva and Yonova, 2002). These compounds show relatively good cytokinin-like activity.

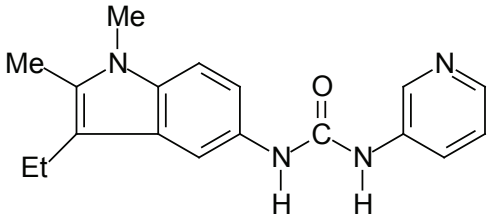
The antioxidant capacity of the thiourea derivatives is not fortuity because the thiourea [-N-C(=S)-NH-] group may turn into an isothiourea group [-N-C(-SH)=N-]. Thus, the compound obtained contains SH-group and may be a reducing agent.

1-Heterocycle-3-(heterocycle)ureas and related derivatives

It is interesting to note that some representatives of this group show important biological properties.

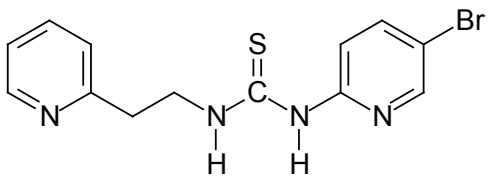
1-Indolyl-3-pyridylureas

They have pharmacological action on depression of migraine, anorexia, Alzheimer's disease, etc. Among them, the most active compound is 1-(3-ethyl-1,2-dimethyl-1H-indol-5-yl)-3-(3-pyridyl) urea (Forbes and Martin, 1992).



1,3-Dipyridylthioureas

These compounds inhibit the replication of HIV and other related viruses. Among them, the most active compound is 1-[2-(2-pyridyl)ethyl]-3-(5-bromo-2-pyridyl)thiourea (Lind et al., 1991).



1,1'-Polymethylenebis(3-cyclohexyl and aryl-substituted)ureas/thioureas

Yonova et al. (1997) and Sergiev (1999) defined a novel series of cytokinin-like compounds – bis-urea/thiourea derivatives of aliphatic diamines containing 2 - 6 methylene groups. These compounds have shown cytokinin activity in four specific bioassays.

ArNHCO-NH(CH_2)nNH-CONHAr, where Ar = phenyl, halogen substituted phenyl, pyridyl, thiazolyl; n=2 - 6 (Yonova et al., 1997).

RNHCO-NH(CH_2)nNH-CONHR, where R= cyclohexyl, phenyl, substituted phenyl; n=2 or 6 (Sergiev, 1999).

All these compounds retarded the dark-induced leaf senescence. It appears that among bis-(phenyl)urea derivatives, more active are the compounds with a longer polymethylene chain while among bis-(4-halogenophenyl)ureas – those with a shorter polymethylene chain.

Possibilities for practical application of synthetic cytokinin-type phytoregulators

Due to the relative ease of synthesis and increased functionality in biological systems, SPGRs are promising candidates for both development of new classes of agrochemicals and multiple applications; at the same time they appear to have the potential to play an important role in the mechanism of action and in the impact on plant tolerance to environmental stress factors.

The exogenous application of cytokinins (natural and synthetic) is based on their major biological effects. In general, practical use of phenylurea cytokinins in agricultural is currently limited.

Cytokinins regulate cell division, growth and development in vitro and in vivo

Cytokinins have a great potential to be employed in plant biotechnological methods that develop very rapidly. This is required by the necessity of quicker and cheaper methods for propagation,

studies on the genetic diversity of plant species and for supplementing of the conventional breeding methods for creation of new cultivars with higher productivity and improved market characteristics.

The basic process during *in vitro* regeneration requires to optimize the desired response by i) selection of expedient starting plant explants and ii) successful introduction of explants into culture. Namely here, the use of growth regulators has very a great significance. The nutrient media must contain cytokinin and an optimal concentration of auxin. The ratio of cytokinin to auxin determines the differentiation of cultured plant tissues to either shoots or roots. Among the cytokinins, the most frequently used are kinetin, benzylaminopurin (BAP), thidiazuron and 4PU-30. In all instances, the biological activity of phenylurea-type cytokinins is higher than or comparable to that of the most active adenine-type cytokinins. The phenylurea derivatives had strong cytokinin-like and organogenic effects on a wide range of species and on species that respond little to adenine-type cytokinins. Further, they can keep high activity during a longer time of exposure in the culture systems than conventional cytokinins.

Nowadays, the micropropagation of many ornamental and ligneous species, forage and technical/industrial crops, medicinal plants, recalcitrant woody species, etc. is a routine practice in many biotechnology laboratories over the world widely employing more active phenylurea cytokinins (Yonova et al., 1989; Lu, 1993; Murthy et al., 1998; Siddique and Anis, 2007; Yonova and Kapchina-Toteva, 2009; Uzunova, 2009).

Treatment of excised *Cucurbita pepo* (zucchini) cotyledons with BAP enhanced significantly cotyledon growth and stimulated protein and RNA synthesis (Ananieva and Ananiev, 1999). Cytological investigations of Stoynova-Bakalova (2008) demonstrated the differential effect of purine- (BAP, zeatin and kinetin) and phenylurea- (4PU-30) cytokinins on the cell growth, cell division and cell enlargement in zucchini cotyledon meristems. BAP and 4PU-30 stimulated strongly both processes while 4PU-30 caused cotyledon enlargement predominantly in length. Similar effects of BAP and 4PU-30 on RNA synthesis in isolated nuclei of *Cucurbita pepo* cotyledons was observed by Ananiev et al. (1987). Both cytokinins stimulated more rapidly RNA-polymerase-I activity and much more slowly and to a lesser extent RNA-polymerase-II activity.

Cytokinins delay leaf senescence

Treatment with phenylurea cytokinin 4PU-30 increased chlorophyll and protein contents as well as photosynthetic activity in maize seedlings and wheat flag leaves (Karanov et al., 1992; Stefanov et al., 1998)

Spraying field grown wheat and maize plants with two novel phenylurea cytokinins 3-CP-2PU and 3-CP-4MPU (synthesized by us) resulted in higher grain yield and biomass weight, respectively. These substances delayed senescence and improved photosynthetic activity of flag leaves at the early grain filling stage and of forage maize leaves (Lazova and Yonova, 2010).

Spraying rice plants with BAP resulted in higher grain yield through maintaining

higher Rubisco level and photosynthetic rates in leaves during the mature period.

- Treatment with BAP and Metribuzin enhanced the area, weight, and specific weight of the expanding leaf as well as the content of chlorophylls, carotenoids, proteins, and Rubisco in sugar beet leaves. It was concluded that cytokinins retard leaf senescence, thus enhancing the photosynthetic activity of a unit leaf area. Qualitatively, Metribuzin and BAP exerted similar effects on the structural and functional characteristics of the photosynthetic apparatus (Chernyad'ev, 2000).

Delayed senescence of cut flowers during vaselife

Ureidotriazolyl-derivatives are assessed as cytokinins and anti-senescence agents. 1-Phenyl-3-(1,2,4-triazol-4-yl)ureas and thioureas can prolong the vaselife of cut carnations with 11 days. High freshness of cut carnations was also achieved using (thio)urea derivatives synthesized in our laboratory, the most active being *para*-phenylthioureidosalicylic acid (Vassilev et al., 1979).

Treatment with Thidiazuron inhibited significantly leaf yellowing and flower abscission in cut tulips, chrysanthemum and phlox. It may be highly useful in improving post-harvest performance of cut flower heads in vases (Sankhla et al., 2003).

Cytokinins regulate the metabolism of important biomolecules, alter sink-source relations

Treatment with Thidiazuron and DPU enhanced total protein content, dry matter and chlorophyll quantity along with an increase in the enzyme activities of PEPC and Rubisco in barley plants (Tsenova, 1988).

Thidiazuron-treatment induced an increase in the activity of carbonic anhydrase and the photosynthetic CO_2 assimilation in soybean plants (Vaklinova et al., 1991).

Single foliar application of two novel phenylurea cytokinins 3-CP-2PU and 3-CP-4MPU on wheat plants caused changes in the polypeptide patterns of soluble proteins from flag leaves. Both substances increased differentially the quantity and composition of some individual polypeptides compared to the non-treated leaves (Lazova and Yonova, 2008).

Foliar application of BAP increased grain protein in four wheat cultivars. It is concluded that the delay of senescence induced by BAP might allow more energy to be available for N uptake by the crop leading to an increase in grain protein (Caldiz et al., 1991).

Two phenylurea cytokinins 3-CP-2PU and 3-CP-4MPU increased also nitrogen content in mature leaves of wheat and maize promoting the accumulation of amino acids and other nitrogen substances (Yonova, unpublished data). Exogenously applied, both urea substances increased grain yield in wheat and photosynthetic potential in maize and sunflower plants (Yonova et al., Bulg. Patent, 1993).

The phenylurea cytokinin 4PU-30 applied in combination with gibberellin to grapevines, caused an increase by 175% (25 ppm), 184% (50 ppm) and 190% (100 ppm) of the weight of berry cluster as compared to gibberellin alone, while the application of BAP resulted in an increase by 122% (100 ppm) and 132% (200 ppm)

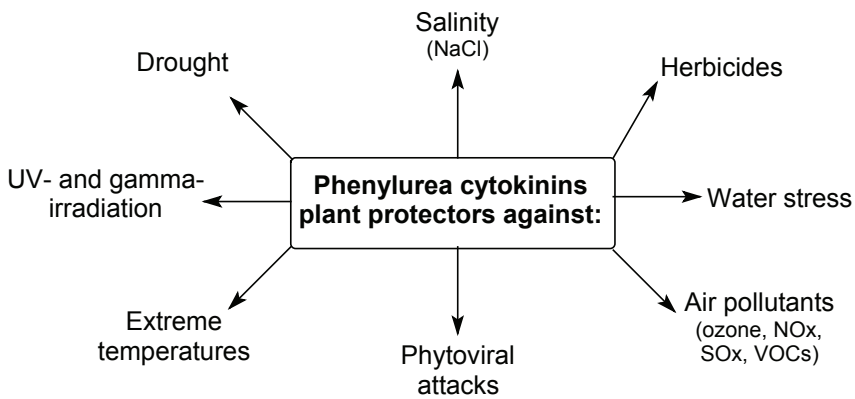
(Isogai, 1981)

Thidiazuron alone or in combination with foliar fertilizer enhanced seed yield and the quantity of active secondary metabolites (silymarin, etc.) in milk thistle (*Silybum marianum* L.) (Geneva et al., 2008).

The phenylurea cytokinin 4PU-30 showed antagonistic effect to the herbicide chlorsulfuronin in relation to the proteolytic activity and protein composition of etiolated maize seedlings (Petkova et al., 2003).

Cytokinins increase plant resistance to environmental stress factors (stress protectors)

To improve stress- and disease-tolerance of cultivated plants, thus increasing crop yield is a crucial problem in agriculture. This problem is usually solved either by classical breeding or producing transgenic plants. Utilization of biologically active substances, controlling the processes of plant adaptation and thus ensuring the favourable property, provides an alternative possibility. In this respect, numerous investigations on the cytokinin-type phytoregulators have been conducted and the results obtained so far are promising. According to many studies, treatment with various natural and synthetic cytokinins can contribute significantly to prevent and reduce the damaging effect of various unfavorable environmental conditions.



Protectors against water stress

Synthetic cytokinins, BAP, TDZ, cartolin-2 and cartolin-4 (the last two substances are Russian preparations) reduced the decrease in chlorophylls, carotenoids, soluble leaf proteins contents, and the carboxylase activity of Rubisco and PEPC in young seedlings and mature leaves of wheat under conditions of progressive water deficiency. Dehydratation caused a more extensive destruction of seedlings compared to leaves of adult plants. The cytokinins played a protective role, increasing the stability of the photosynthetic apparatus. Preparations of cartolins displayed the maximum protective effect (Chernyad'ev, 2005; Monakhova and Chernyad'ev, 2004).

Spraying bean plants with phenylurea cytokinin 4PU-30 before and after water stress alleviated the negative stress effect on the lipid membrane composition permitting plants to resist the harmful environment (Ivanova et al., 1998). The cytokinins BAP and 4PU-30 increased the

photochemical (Hill reaction) activity and thermoluminescence "B"-band in control, water stressed and rehydrated bean plants, but the oxygen flash yields were affected only in the stressed and rehydrated plants (Metwally et al., 1997).

The high active phenylurea cytokinin 4PU-30 overcame partially the drought-induced damage in maize seedlings (Todorov et al., 1998).

The influence of drought and high temperature applied separately or in combination on the photosynthetic activity of young bean plants was investigated. The positive effect of 4PU-30 was well expressed only in under drought conditions (Yordanov et al., 1999).

Protectors against air pollutants (O_3, SO_2, NO_2)

Tropospheric ozone (O_3) is one of the secondary air pollutants, predominantly formed by photochemical reactions involving precursors like nitrogen oxides (NOx) and volatile organic compounds (VOCs) generated by anthropogenic activities. Tropospheric O_3 has a negative impact on growth, development and productivity of crops. Effects of O_3 are observed in a wide range of physiological characteristics, such as accelerated senescence, decreased photosynthetic assimilation, decreased productivity, and reduced carbon allocation to roots.

A cytokinin-like substance**,** 1-[2-(2-oxo-1-imidizolidinyl)ethyl]-3-phenylurea (Ethylenediurea, EDU) was proved as an antiozonant in a number of plant species **(**Lee and Chen, 1982). EDU is one of the most successful and widely used protective chemicals for assessing crop loss from O_3 under ambient field conditions. EDU is known to suppress acute and chronic O_3 injury on a variety of sensitive plants. Mung bean plants (*Vigna radiata* cv Malviya Jyoti) grown at different sites having variable concentrations of SO_2, NO_2 and O_3 showed that the maximum protection by EDU treatment occurred at sites showing higher concentrations of O_3. Complete protection in crop yield was not observed because SO_2 and NO_2 were also present. Experiments with EDU indicated that O_3 can cause significant negative effects on the yield of snap bean, tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and wheat *(Triticum aestivum)* cultivars (Miller et al., 1994; Lee et al., 1997; Singh et al., 2010).

Protectors against UV- and gamma irradiation

Ethylenediurea (EDU) ameliorated substantially foliar damage induced by UV-B radiation in soybean (*Glycine max* L.) (Middleton et al., 2005) but it was not a protector against the UV-B damage in cucumber leaves (Krizek et al., 2001).

The thiourea derivative 1-(4-fluorophenylthiocarbamoyl)-4-methyl-piperazine (FTMP) showing weak cytokinin-like activity, possessed a protective effect against UV-C exposure of barley leaves and human lymphocytes but it was not a protector against the UV-C damage in pea leaves (Gateva et al., 2006).

The search for new more effective radioprotectors is of great theoretical and practical importance for radiation protection in the epoch of atomic energy. Our investigations during the last two decades resulted in the discovery of some synthetic urea and thiourea compounds exhibiting a considerable radio-protective effect. The novel protectors are applied

as post-radiation treatment of pea and soyabean seeds (50-80 Gy) and of oat plants during the vegetation period (6 Gy). These compounds could be applied in the radiation mutagenesis when sublethal doses are used and in the radiation protection of plants due to the increased recombinant potential of irradiated cells. The application of some compounds to increase the radiation protection can lead to new mutations bearing valuable agronomical qualities, such as earlier ripening, increased number of pods, larger seeds, more resistant plants to lodge, etc. (Mehandjiev et al., 1993; Bineva et al., 2006).

Protectors against herbicides (herbicide safeners)

Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by physiological or molecular mechanisms without compromising weed control efficacy. The safener-mediated induction of herbicide-detoxifying enzymes appears to be part of a general stress response.

The phenylurea cytokinin 4PU-30 alleviated to some extent the detrimental effects of the herbicides chlorsulfuron and glyphosate (roundup) in young maize plants (Georgiev and Iliev, 2000; Sergiev et al., 2006). However, 4PU-30 was not a protector against other herbicides such as paraquat, fuzilad, flex, agil (Georgiev, 1996) and even enhanced to some extent the herbicidal effect of paraquat in pea plants (Sergiev et al., 2003).

The thiourea derivative 1-(4-fluorophenylthiocarbamoyl)-4-methyl-piperazine (FTMP) was an effective antidote to chlorsulfuron and paraquat in maize and barley, respectively (Stoilkova and Yonova, 2007; Yonova et al., 2009).

Another phenylurea derivative 1-(3-chlorophenyl)-3-(5-chloro-2-pyridyl)urea (showed high cytokinin-like activity) provided the protection of tobacco callus cultures and young tobacco plants against injury provoked by chlorsulfuron (Zozikova et al., 2006).

Protectors against salinity stress (NaCl)

The urea derivatives 4PU-30 and 1-(2-chloroethyl)-3-(4-methyl-2-pyridyl) urea (with high cytokinin-like activity) manifested a protective effect against NaCl (up to 170 mM) in tobacco callus tissues. The protective potential of the second substance was similar to 4PU-30 up to 85.5 mM of NaCl and equal to that of kinetin up to 126.5 mM of NaCl (Nedjalkova, 2001).

Treatment with 1-(3-chlorophenyl)-3-(5-chloro-2-pyridyl)urea resulted in enhanced tolerance of young tobacco plants to NaCl (85.5 mM). The protective effect was organ-specific, being more strongly pronounced on shoots than on roots (Yonova and Zozikova, Annual report, 2009).

Treatment with Thidiazuron enhanced the tolerance and yield of wheat plants subjected to salinity (Beckett and Van Staden, 1992). Thidiazuron significantly increased the yield of highly stressed wheat plants. In plants treated with high concentrations of TDZ an increase in grain weight more than compensated for the lower grain number was observed.

Protectors against extreme temperatures

The effects of water deficit and high temperature applied separately or in combination on young bean plants were

investigated and a protective effect of 4PU-30 was established (Yordanov et al., 1997).

Protectors against clastogenic action of chemical and physical agents

Recent investigations have shown that some chemical substances possessing weak or no cytokinin activity can modify the defense response against the damaging effects of various chemical (antropogenic genotoxicants) and physical (ionizing radiation) environmental agents. Due to the fact that chemical and physical agents possess a mutagenic effect, the elimination of this negative effect by means of anti-mutagenic substances appears to be very important.

The anti-mutagenic effect of 1,2-di[(2-chloroethyl)ureido]ethan in *Saccharomyces cerevisiae* yeast PV_3 strain against $CdCl_2$ was established. Having in mind that the PV_3 strain had a violated reparative system the authors concluded that this substance had a repairing role (Pesheva et al., 2002).

Pre-treatment of barley root tip meristem cells and cultured lymphocyte cells with synthetic bis-urea 1,1'-hexamethylenebis[3-(3,5-dichloro-4-pyridyl)] urea (showing a strong senescence-delaying activity in barley leaf segments) for 1 h decreased significantly the frequency of chromatid aberrations induced by the clastogenic effect of hydroxyurea and N-methyl-N-nitro-N'-nitroso-guanidine (Chankova et al., 2001). However, the synthetic bis-urea 1,1'-propylenebis[3-(4-chlorophenyl)]urea did not show the ability to reduce the frequency of chromatid aberrations induced by UV-C radiation of *Hordeum vulgare* root tip meristem cells (Jovtchev et al., 2003).

Protectors against phytopathogen attack

High antiphytoviral activity of some 1,3-disubstituted ureas with cytokinin activity was established. The compounds tested inhibited the replication of potato virus X (PVX) and red clower mottle virus (RCMV) inoculated in leaves of *Nicotiana tabacum* L., cv. Samsun NN and *Pisum sativum* L., cv. Nadja, respectively. The inhibitory effects were higher in secondarily infected leaves than in the inoculated ones. Therefore, it may be suggested that these compounds depress some steps of the replication cycle or the phloem transport of viruses rather than the process of infection. The same structure - activity relationships for antiphytoviral and cytokinin activities of these compounds were observed (Yonova et al., 1992).

Protectors against heavy metals

The phenylurea cytokinin 4PU-30 (10^{-5}M) definitely counteracted the heavy metal-caused inhibition of the growth while the effects of the purine cytokinin BAP (10^{-5}M) were contradictory. In the marginal region of cotyledon plate meristem, BAP even enhanced the inhibitory effect of Cu^{2+}, especially toward the division activity. The counteracting effects of cytokinins to Cu^{2+} in excess were better expressed in the stress light conditions for the cotyledon growth (continuous light or complete darkness) (Stoynova-Bakalova and Petrov, 2009).

The phenylurea cytokinin 4PU-30 generally increased flavonol accumulation to the greatest extent, and reduced Cu^{2+}-induced inhibition of both cotyledon growth and flavonol glycoside

accumulation in zucchini cotyledons (Stoynova-Bakalova et al., 2009).

Ultrastructural disturbances in the chloroplast structure in primary leaves of bean plants treated with $CuSO_4$ were found on the 120th h in the light. These disturbances decreased significantly when the exogenous cytokinin kinetin was applied (Stoynova et al., 1991).

Other functions of phenylurea cytokinins

Phenyl(thio)urea compounds have focused considerable attention because of other activities such as pesticidal (herbicidal, fungicidal, insecticidal, acaricidal, nematocidal, etc.); defoliating effects (thidiazuron, developed as a cotton defoliant); plant growth regulators in controlling fruit growth and partenocarpic fruit formation; pharmacological properties (antiaterosclerotics, antioxidants, antiarrhythmia, antihypertensive, antituberculous, antiviral, antimalarial, antitumor, antileukemic agents) etc.

Evidence exist that 4PU-30 interacts positively with naturally occurring cytokinins in stimulating fruit growth of kiwifruit, suggesting a possible protective role of 4PU-30. These results are discussed in terms of the interaction and mechanism of action of plant growth regulators in controlling fruit growth (Woolley and Currie, 2005).

Hydrohalogenides of 1-phenyl-3-(polymethylene-acethylen)bis-ureas $[PhNHCONH(CH_2)nC{\equiv}C]_2.mHX$, showed beneficial property as indicators of registration of the stress impact of the environment – high temperature, radiation, increased moisture (Preziosi and Prusik, 1989).

Antiproliferative properties of 1,3-dipyridyl-thiourea derivatives on HIV and related viruses were found (Lind et al., 1991).

A large number of synthetic 1-(un- and substituted-phenyl)-3-(5-substituted-2-thiazolyl)ureas and 3(5)-acylaminopyrazole derivatives have shown potent inhibitory activity on cell proliferation of different tumor forms. The compounds are active as cdk/cyclin inhibitors at a concentration of 0.56 μmol (Villa et al., 2000; Prevarello et al., 2001).

Phenyl(thio)urea cytokinins possess some important properties which predetermine the enhanced interest for future use

These compounds show some properties like 1) resistance to oxidases; 2) stability; 3) biological activity at lower concentrations and for longer time-period than the adenine-type cytokinins; 4) considerably less expensive and toxic; 5) because *phenylurea cytokinins* are not structurally analogues to the purine bases, they are not involved in the nucleic acids metabolism as antimetabolites. Thus, this type of cytokinins is more harmless for humans than the purine cytokinins.

The prospect of the above-listed properties of phenylurea cytokinins may render them as particularly valuable as sources of cytokinins in tissue culture systems where degradation of growth regulators is a limiting factor. In addition, these compounds are valuable as active ingredients in many useful agrochemical and pharmacological preparations.

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