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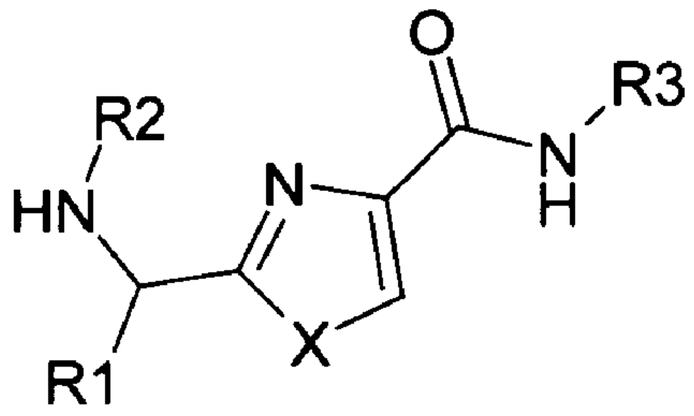
(54) Titre : NOUVEAUX AMIDES D'ACIDE AMINOALKYL-OXAZOLCARBOXYXLIQUE ET D'ACIDE AMINOALKYL-THIAZOLCARBOXYLIQUE COMME SUBSTANCES FAVORISANT LA REGENERATION POUR LES ORGANES SENSORIELS ET LES TISSUS POSTMITOTIQUES

(54) Title: NOVEL AMINOALKYLOXAZOLE- AND AMINOALKYLTHIAZOL CARBOXYLIC ACID AMIDES AS REGENERATION-PROMOTING SUBSTANCES FOR SENSORY ORGANS AND POST-MITOTIC TISSUE



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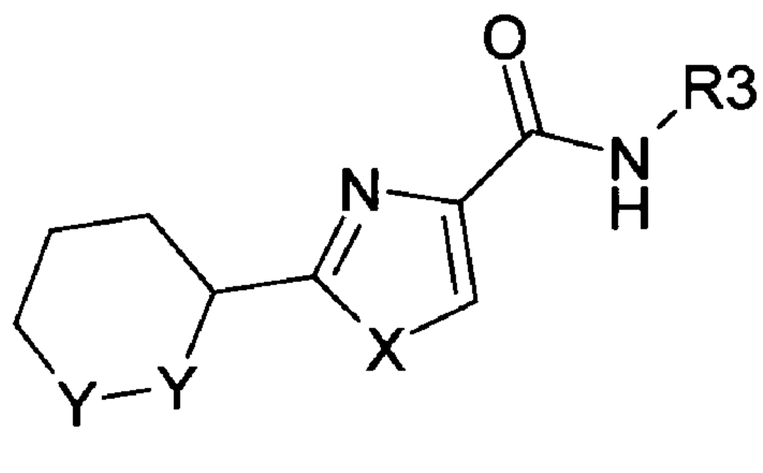

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X = O, S

(57) Abrégé/Abstract:
The present invention relates to novel aminoalkyl-oxazole and aminoalkyl-thiazole carboxylic acid amides that stimulate the endogenous regeneration of terminally differentiated cells in highly specialized organs, tissues and sensory epithelia in mammals in

CA 2789013 A1 2011/08/11

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(57) Abrégé(suite)/Abstract(continued):
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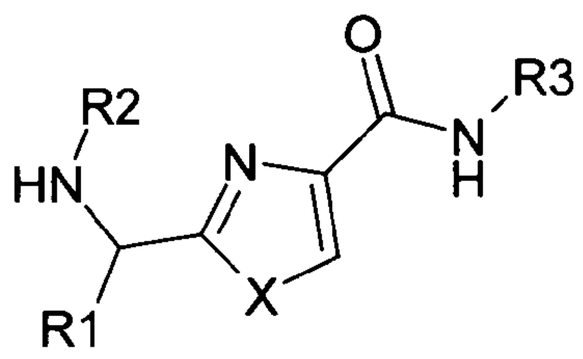
Erklärungen gemäß Regel 4.17:

[Fortsetzung auf der nächsten Seite]

(54) Title: NOVEL AMINOALKYL-OXAZOLE AND AMINOALKYL-THIAZOLE CARBOXYLIC ACID AMIDES AS REGENERATION-PROMOTING SUBSTANCES FOR SENSORY ORGANS AND POST-MITOTIC TISSUE

(54) Bezeichnung : NEUE AMINOALKYL-OXAZOL- UND AMINOALKYL-THIAZOLCARBONSÄUREAMIDE ALS REGENERATIONSFÖRDERNDE SUBSTANZEN FÜR SINNESORGANE UND POSTMITOTISCHE GEWEBE



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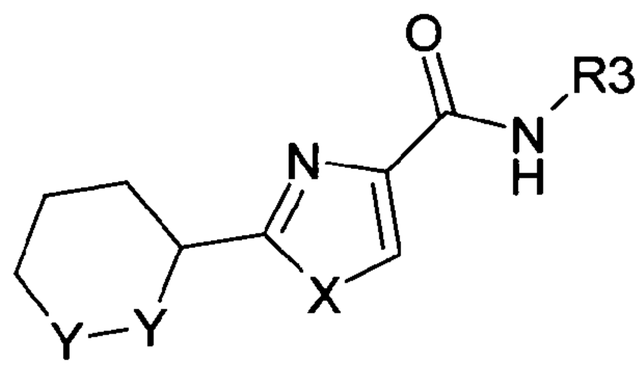


(2)

X = O, S

(57) Abstract: The present invention relates to novel aminoalkyl-oxazole and aminoalkyl-thiazole carboxylic acid amides that stimulate the endogenous regeneration of terminally differentiated cells in highly specialized organs, tissues and sensory epithelia in mammals *in situ*. The claimed low-molecular-weight compounds are able to induce corresponding cell biological changes such as dedifferentiation, proliferation and the subsequent terminal redifferentiation of cells of the normally post-mitotic tissue. The invention in particular relates to compounds with which a *de novo* formation of hair sensory cells in the organ of Corti can be obtained by inducing cell separation of supporting cells of the inner ear and hearing can be restored after hair cell loss. The compounds according to the invention for the first time enable a causal treatment of inner ear hardness of hearing caused, for example, by noise, ototoxic substances, symptoms of old age or genetic causes on the basis of regenerative biology. The invention further relates to methods for producing the compounds according to the invention, to the formulation (1) (2) thereof as pharmaceutical preparations and to the use thereof for producing pharmaceuticals for regenerative medicine.

(57) Zusammenfassung:

[Fortsetzung auf der nächsten Seite]

WO 2011/095338 A1

WO 2011/095338 A1

— *hinsichtlich der Berechtigung des Anmelders, ein Patent zu beantragen und zu erhalten (Regel 4.17 Ziffer ii)*

— *Erfindererklärung (Regel 4.17 Ziffer iv)*

Veröffentlicht:

— *mit internationalem Recherchenbericht (Artikel 21 Absatz 3)*

Die vorliegende Erfindung betrifft neue Aminoalkyl-oxazol- und Aminoalkyl-thiazolcarbonsäureamide, die die endogene Regeneration von terminal differenzierten Zellen in hochspezialisierten Organen, Geweben und Sinnesepithelien beim Säuger in situ stimulieren. Die beanspruchten niedermolekularen Verbindungen sind in der Lage, entsprechende zellbiologische Veränderungen wie die Dedifferenzierung, die Proliferation und die nachfolgende terminale Redifferenzierung von Zellen des normalerweise postmitotischen Gewebes zu induzieren. Im Besonderen betrifft die Erfindung Verbindungen, mit denen durch eine Induktion der Zellteilung von Stützzellen des Innenohres eine de novo Bildung von Haarsinneszellen im Corti'schen Organ erreicht und das Hörvermögen nach Haarzellverlust wiederhergestellt werden kann. Die erfindungsgemäßen Verbindungen ermöglichen erstmals eine kausale Behandlung der beispielsweise durch Lärm, ototoxische Substanzen, Alterserscheinungen oder genetische Ursachen bedingten Innenohrschwerhörigkeit auf regenerationsbiologischer Grundlage. Die Erfindung betrifft weiterhin Verfahren zur Herstellung der erfindungsgemäßen Verbindungen, zu deren Formulierung (1) (2) als pharmazeutische Präparate sowie ihre Anwendung zur Herstellung von Arzneimitteln für die regenerative Medizin.

Description

Novel aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides as regeneration-promoting substances for sensory organs and post-mitotic tissue

The present invention relates to novel aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides that stimulate the endogenous regeneration of terminally differentiated cells in highly specialized organs, tissues and sensory epithelia in mammals *in situ*.

The claimed low-molecular-weight compounds are able to induce corresponding cell biological changes such as dedifferentiation, proliferation and the subsequent terminal redifferentiation of cells of the normally post-mitotic tissue.

The invention in particular relates to compounds with which a *de novo* formation of hair sensory cells in the organ of Corti can be obtained by inducing cell separation of supportive cells of the inner ear and hearing can be restored after hair cell loss.

The invention further relates to processes for preparing the compounds according to the invention, to the formulation thereof as pharmaceutical preparations and to the use thereof for producing pharmaceuticals for regenerative medicine.

Prior art

In virtually all professional and social spheres, impaired hearing and thus a restricted ability to communicate have considerable implications on the quality of life. The sensory disorder "hardness of

hearing" is one of the most urgent health problems in a society depending on communication.

On average, hardness of hearing affects about 10% of the population of the industrialized nations. In Germany, there are estimated to be 16 million people who are hard of hearing, almost one fifth of the total population (ifo-Institut, 1986). Thus, hardness of hearing is not only the most frequent disorder of a sensory organ but also one of the most frequent chronic disorders in general.

Looking at the causes of hardness of hearing, in about 80% of the cases the people affected are suffering from sensorineural hearing loss. One of the most frequent reasons for this is the loss of sensory hair cells in the organ of Corti, the auditory sensory epithelium which, owing to exposition to noise, side-effects of ototoxic medicaments, age-related degeneration or genetic causes are lost irreversibly by cell death (Nadol, 1993).

Hitherto, only the prosthetic provision of hearing aids can be offered for this most frequent form of hearing loss. However, for the persons affected the result of this provision is often unsatisfactory, owing to the lack of speech recognition. Accordingly, hearing aids are actually used by only a relatively small proportion of the hard of hearing.

To date, there is no curative medical treatment option for the main cause of sensorineural hearing loss. Such a causal treatment would be possible only by replacing or regenerating the lost sensory hair cells of the organ of Corti.

In most mammalian organs and tissues, the ability for regeneration after damage is limited, or not present at all. Only very few organs and tissues such as, for example, liver, bones or skin have, over the entire lifetime of the organism, the ability for spontaneous regeneration by forming new cells. In many cases, the corresponding cells in the highly specialized organs and tissues (for example heart, brain, skeletal muscle or the sensory epithelia of eye and inner ear) leave the cell cycle irreversibly to remain in a terminally differentiated state. As a consequence, these tissues have also lost their ability for spontaneous regeneration in the case of damaging events happening. Accordingly, this leads to irreversible functional deficits. Thus, for example, a myocardial infarct in the case of the heart or a stroke in the case of the brain often means irreversible damage of the tissue areas affected, with corresponding permanent loss of function.

There are, however, for example in amphibians, tissues and organs, exemplified by retina and extremities, where there is terminal differentiation, but which are nevertheless capable of spontaneous *in vivo* regeneration (Tsonis, 2000; Tsonis, 2002). The central cell biological event in these examples is cellular dedifferentiation, which allows the generation of multipotent precursor cells from which regenerated cells may be formed by proliferation and redifferentiation.

Thus, dedifferentiation plays the decisive role in the regeneration of terminally differentiated tissue of amphibious animals. In contrast, other vertebrates have low regeneration abilities.

Until about 20 years ago, for the hearing organ of mammals and birds, it was assumed that the sensory hair cells in the inner ear can be formed only during a short critical phase of embryonic development (Ruben, 1967). After this phase, the sensory epithelia were thought to be postmitotic and therefore not able to regenerate their sensory cells. However, surprisingly it was discovered that, after acoustic trauma and ototoxic damage, avian cochlea are capable of spontaneously regenerating sensory hair cells (Cotanche 1987; Cruz et al., 1987).

Cell division of supportive cells directly adjacent to the destroyed sensory hair cells has been described as the basic biological mechanism for sensory hair cell regeneration in avian cochlea (Corwin and Cotanche, 1988; Ryals and Rubel, 1988), where a population of undifferentiated cells is formed which are capable of redifferentiation to newly formed sensory hair cells and supportive cells. The result is the virtually complete morphological and functional recovery of the sensory epithelium in birds (Cotanche, 1999; Smolders, 1999).

Although this was initially obvious, owing to fundamental cell biological differences it has hitherto not been possible to apply findings from other models to mammals.

Corresponding experiments concerning the regeneration of sensory hair cells in mammals gave no (Roberson and Rubel, 1994; Vago et al., 1998; Daudet et al., 1998; Daudet et al., 2002; Yamasoba et al., 2003) or very few (Yamasoba and Kondo, 2006) indications of a capability for spontaneous cell division of supportive cells in the organ of Corti. In particular, even after administration of growth factors, there are no

indications of an inducible proliferation of supportive cells in the organ of Corti (Staecker et al., 1995; Daudet et al., 2002). Various experiments with cultures of early development stages of the organ of Corti of embryonal mice likewise showed, after defined laser damage, only individual proliferative events (Kelley et al., 1995).

This total lack of cell divisions suggests that the highly specialized supportive cell populations in the normal adult organ of Corti have reached a terminally differentiated state and are unable to re-enter the cell cycle. Thus, in the case of the inner ear, even a single acoustic trauma may result in the destruction of sensory hair cells, followed by unavoidable and irreversible loss of hearing.

It had recently been found that an extract can be obtained from amphibian tissues (for example extremities) undergoing regeneration which is capable of inducing dedifferentiation even in mammalian cells (McGann et al., 2001). Using this extract, with appropriate stimulation, the dedifferentiation-based mechanism for the regeneration of terminally differentiated cells can be transferred from amphibians to mammals (Odelberg, 2002). However, the regeneration extracts mentioned are "protein cocktails", and details with regard to their composition are not known.

However, in the meantime it has also been possible to achieve a corresponding effect in mammalian muscle cells using a defined low-molecular-weight compound (Chen et al., 2004). By screening, is was furthermore possible to identify several low-molecular-weight compounds producing regeneration biology-relevant effects in various cell types including glia cells (reviews in Xu et al., 2008; Schugar et al., 2008; Feng

et al., 2009; Li and Ding, 2009). These effects suggest that it may also be possible to induce dedifferentiation-based regeneration in further cell types using suitable low-molecular-weight compounds (Tsonis, 2004; Kim et al., 2004; Odelberg, 2002).

Hitherto, the transfer of this concept to regeneration biological studies on the inner ear has been unique. Low-molecular-weight compounds capable of effecting sensory hair cell regeneration in the inner ear are neither known nor patented.

As yet, other concepts pursued in the current art for regenerating sensory hair cells in the organ of Corti likewise show little promise with regard to clinical application.

In the modulation of cell cycle regulation of supportive cells by switching off the cell cycle inhibitor $p27^{Kip1}$, it was possible to achieve cell divisions *in vivo* (WO 99/42088). However, differentiation to sensory hair cells has hitherto only been observed under *in vitro* conditions outside of a tissue context (White et al., 2006).

In an *in vivo* model with induced sensory hair cell loss, gene therapeutically induced transdifferentiation of supportive cells with the transcription factor *Math1*, essential for sensory hair cell differentiation, led to the conversion of supportive cells into sensory hair cells, even with partial functional recovery of the organ function (Izumikawa et al., 2005; Kawamoto et al., 2003). However, the reduction in the number of supportive cells resulted in functional limitations for the organ of Corti, as normal functioning of the complex micromechanic in the transduction process is impossible without supportive cells.

On activation of endogenous progenitor stem cells residing in the organ or of exogenous administration of heterologous stem cells to the inner ear, promising results were obtained (Tateya et al., 2003; Naito et al., 2004; Martinez-Monedero et al., 2007a, b; Li et al., 2003). However, a targeted or functionally relevant transplantation of stem cells into the inner ear has hitherto not been realized.

Objects

Accordingly, it was an object of the present invention to identify low-molecular-weight compounds which stimulate an endogenous regeneration of terminally differentiated cells in highly specialized organs, tissues and sensory epithelia in mammals *in situ*. In particular, these compounds should allow restoration of hearing in mammals by *de novo* formation of sensory hair cells in the adult organ of Corti. Based on these compounds, it should be possible, for the first time, to treat the causes of inner ear hardness of hearing on the basis of a pharmaceutical having regeneration biological activity.

The object is achieved by providing novel low-molecular weight aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides having regeneration-promoting properties, as shown in claim 1. Preferred embodiments of these compounds are declared in claim 2. Claims 3 to 5 relate to the use of the compounds according to the invention in medical therapy. Claim 6 relates to processes for preparing the compounds according to the invention, and claims 7 to 8 relate to pharmaceutical preparations comprising the compounds according to the invention, and to their preparation. Claims 10 and 11 disclose the use of the

compounds according to the invention and pharmaceutical preparations for treating certain disorders in mammals, and claims 12 and 13 disclose the corresponding therapeutic procedures.

By reference, the wording of all claims is herewith incorporated into the description.

The essential feature of the present invention consists in the fact that the compounds according to the invention provide, for the first time, structurally defined chemically active compounds which can effect regeneration of terminally differentiated cells in mammals, in particular of sensory hair cells in the mammalian inner ear. For this reason, the present invention has a unique feature by identifying novel, hitherto unknown low-molecular-weight compounds capable of stimulating regeneration biologically relevant processes such as dedifferentiation, proliferation and the resulting regeneration of cells from normally postmitotic tissues.

The invention furthermore comprises the utilization of the regeneration-promoting properties of the compounds according to the invention for causal treatment of inner ear hardness of hearing after damage and loss of the sensory hair cells in the organ of Corti up to complete recovery of hearing in humans and animals.

Detailed description

Owing to the complex tissue structure in the inner ear, from among the various methods of regenerative medicine, only endogenous regeneration appears to be feasible as regeneration *in situ*. A precondition of the induction of this regeneration of the sensory hair cells is the suitable stimulation of the normally

highly differentiated postmitotic auditory sensory epithelium. Target cells are the supportive cells, which are directly adjacent to the damaged cells and which may serve as potential precursors for a reformation of sensory hair cells.

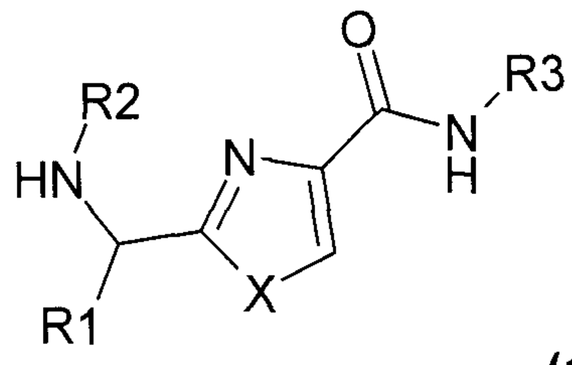
Although, based on the current state of the art, it can be assumed that the regeneration biological mechanism of the dedifferentiation of cells can be triggered by certain active compounds, attempts to identify corresponding compounds for regeneration in the inner ear have hitherto been unsuccessful.

The ability of organic compounds to trigger a regeneration biologically relevant effect in the inner ear is target structure-dependent and impossible to predict accurately.

In addition to potentially active structural features, a decisive factor for the prospect of success in the biological tests is the selection of a suitable scaffold having active compound potential depending on and with reference to the target system.

By extensive screening of compounds with potential regeneration-promoting properties from various compound classes, it was surprisingly possible to identify low-molecular-weight compounds capable of inducing, *in vitro* and *in vivo*, corresponding cell biological changes such as dedifferentiation and subsequent proliferation of otic supportive cells up to the regeneration of sensory hair cells. Using lead structure optimization, it was possible to develop structural analogs of these compounds having superior regeneration-promoting activity in mammals.

Accordingly, the present invention relates to aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides of the formulae (1) and (2)



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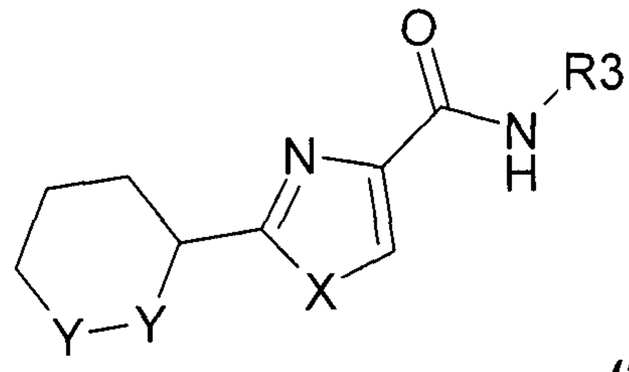

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and to their use as active compounds for stimulating the endogenous *in situ* regeneration of terminally differentiated cells in highly specialized organs and tissues, preferably the brain, the heart, the skeletal muscles and particularly preferably sensory epithelia.

Here,

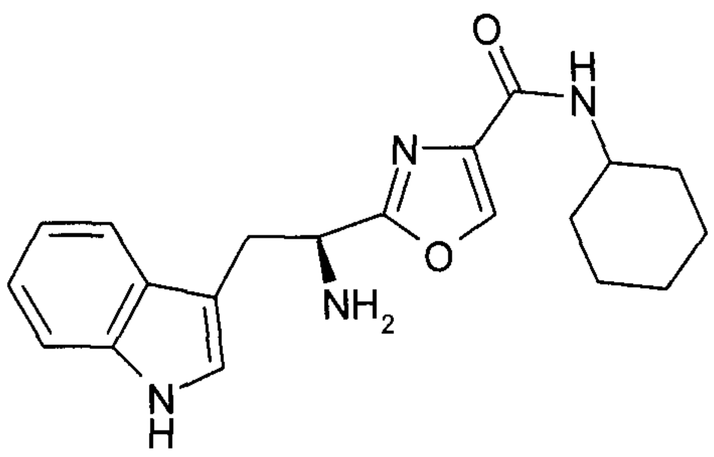
X represents O or S,

Y represents C or N, where the two atoms must be different from one another,

R2 represents hydrogen or acyl and

R1 and R3, which may be identical or different, represent a substituent selected from the groups below: branched or straight-chain, substituted or unsubstituted alkyl groups, alkylcycloalkyl groups, alkylaryl groups, cycloalkyl groups, cycloalkylaryl groups, aryl groups and arylcycloalkyl groups which optionally contain heteroatoms, where R1 preferably represents (1H-indol-3-yl)ethyl and R3 preferably represents cyclohexyl.

A preferred embodiment relates to aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention which correspond to the formulae (3) to (8) below.



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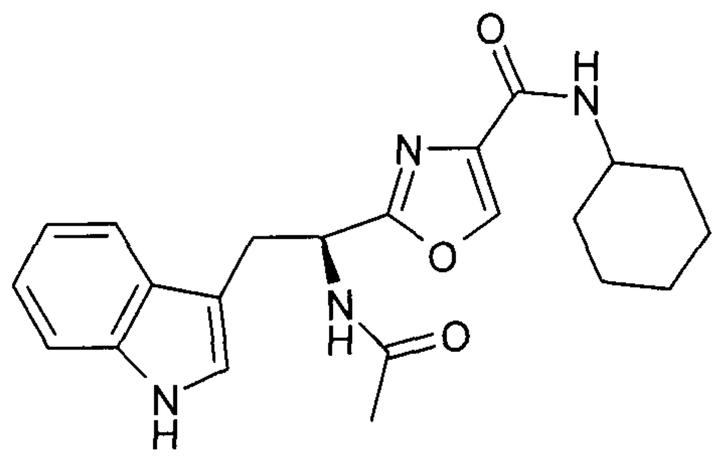

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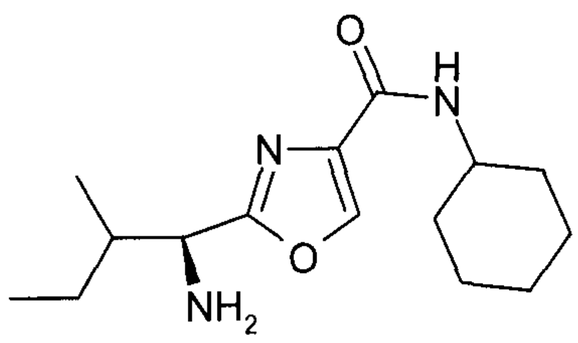

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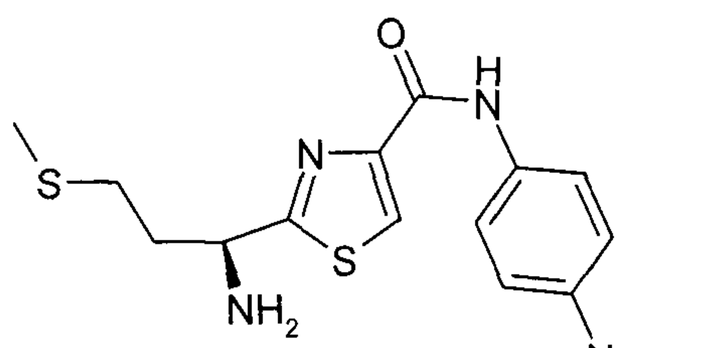

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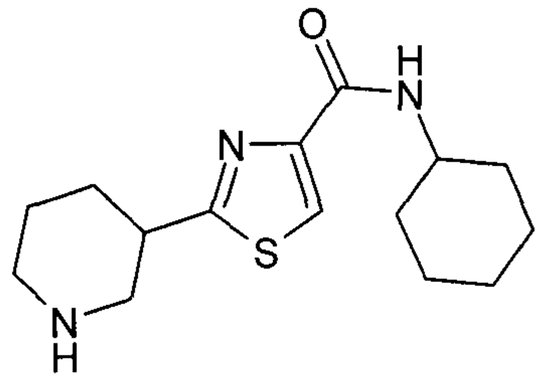

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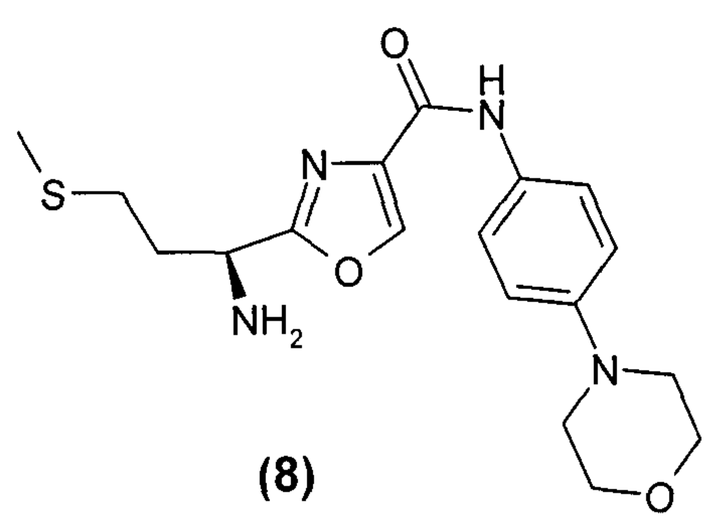


(8)

These definitions include pharmaceutically acceptable salts (preferably non-toxic and physiologically tolerated salts), the stereoisomers, stereoisomer mixtures, all tautomers, prodrug compounds, preferably prodrug esters or prodrug peptides, and mixtures of the compounds according to the invention of the formulae (1) to (8).

The potential of the low-molecular-weight aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention for dedifferentiating cells which are already differentiated was demonstrated in a cell culture assay

using stem cells, isolated from the inner ear of mice, which have otic development potential and, in cell culture, were differentiated to epithelium islands relatively similar to the original sensory epithelium. Compared to the control, the proportion of *Sox2*-expressing cells, a stem cell marker which is also expressed during ontogenesis of the organ of Corti and which is accepted as an indicator of cellular dedifferentiation, could be more than doubled by administration of the compounds according to the invention. With a proportion of up to 7.4% *BrdU (bromodeoxyuridine)*-positive cells, as indicator of cellular proliferation, the activity of the compounds according to the invention differs significantly from that of the differentiation and DMSO controls where no *BrdU* is incorporated.

It was thus possible to demonstrate the surprising biological activity of the compounds according to the invention in the sense of the stimulation of dedifferentiation and subsequent proliferation of differentiated otic cells in an *in vitro* model.

Immunocytochemical analysis of the stem cell markers on the dedifferentiated epithelium confirmed the assumption that the compounds according to the invention, after accumulation in the nucleus, first induce dedifferentiation of differentiated cells in the sense of reprogramming and then facilitate their proliferation.

In the cell culture model, by dose/activity analysis of the compounds according to the invention, the concentration range for effective activity with regard to proliferation was defined to be in the range of from 0.1 µM to 100 µM, preferably from 1 µM to 3 µM, of substance in the cell culture medium.

Effects comparable to that in the cell culture assay were also achieved in the *in vitro* organ culture of a native organ of the mouse, i.e. in the complex cellular structure of the organ of Corti. After ototoxic damage of the sensory hair cells by the aminoglycoside antibiotic neomycin, administration of the compounds according to the invention resulted in *in situ* proliferation of various supportive cells in the organ of Corti, such as the laterally orientated Deiter's cells (outer phalangeal cells) and outer border cells in the region of the outer hair cells, the inner phalangeal cells and inner border cells in the region of the inner hair cells and also other supportive cells thought to be potential precursor cells for the regeneration of inner sensory hair cells. In detail, it was possible to demonstrate individual labelled nuclei in various stages of mitosis including completed cell division with two nuclei present.

In the *in vivo* model of the adult guinea pig, immunohistochemical findings additionally indicated regeneration, induced by the compounds according to the invention, of sensory hair cells based on cell divisions of supportive cells, preferably in the regions of the organ of Corti damaged by acoustic traumata. In corresponding control animals and control organs without administration of the compounds, no spontaneously regenerated hair cells were found.

In the *in vitro* and *in vivo* tests carried out to date, toxic effects or incompatibilities of the compounds according to the invention have not been observed at the relevant concentration ranges.

The invention furthermore provides the preparation of the aminoalkyloxazole- and aminoalkylthiazolecarboxylic

acid amides according to the invention which, according to the invention, can be employed as active compounds for stimulating endogenous *in situ* regeneration of terminally differentiated cells in highly specialized organs and tissues, preferably the brain, the heart, the skeletal muscles and particularly preferably sensory epithelia.

The synthesis of the compounds according to the invention is carried out in a number of steps. Detailed synthesis procedures can be found in Working Example A. The starting materials for preparing the compounds according to the invention are known and can be obtained from the specialist trade or be prepared by known procedures.

One possible route is the synthesis of the compounds according to the invention on a solid phase starting with an amino acid amide. To this end, the amino acid, the side chain of which represents substituent R1, is immobilized on a polymeric support having an activated carbonic ester group, and its acid group is then amidated. The cyclization of the amino acid amide to the oxazole or of the amino acid thioamide to the thiazole is carried out using bromopyruvic acid and N,N-dimethylaniline. If ethyl bromopyruvate is employed, the ester group formed has to be hydrolyzed afterwards. For the synthesis of the thiazoles, the amino acid amides are converted prior to the cyclization into the corresponding thioamides using Lawesson's reagent. The amino acid oxazole/thiazolecarboxylic acids obtained are then reacted with an amine representing the substituent R3. Finally the amino acid oxazole/thiazolecarboxylic acid amides are cleaved with acid from the synthesis resin, followed by chromatographic purification.

In a further preparation variant, the synthesis of the compounds according to the invention is carried out in solution. The synthesis takes place using protective group chemistry starting with a Boc-protected amino acid having the side chain R1. In principle, the steps of the synthesis correspond to those described for the solid phase. At the end of the synthesis, the Boc protective group is removed with acid, followed by chromatographic purification.

Both preparation processes permit the synthesis both of racemic and of enantiomerically pure compounds. The compounds are obtained either in free form or as a salt, provided salt-forming groups are present. Preferred salts of the compounds according to the invention are pharmaceutically acceptable salts. Examples of such salts are salts of inorganic or organic acids, salts of inorganic or organic bases and salts of basic or acidic amino acids.

The compounds according to the invention can also be modified as prodrug compounds, preferably as prodrug esters or prodrug peptides, or the like. In certain cases, by coupling cell penetration-enhancing molecules such as, for example, biotin or maleimidopropionic acid, optionally via suitable spacer molecules, to the primary amino group, or by acylation of this amino group, corresponding to substituent R2, it is possible to improve the bioavailability and thus the efficacy of the compounds according to the invention.

The invention furthermore provides the use of the aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention for preparing pharmaceutical preparations for treating disorders in mammals associated with damaged postmitotic tissues, in particular for treating inner ear hardness of hearing

in mammals caused by damage and loss of sensory hair cells in the organ of Corti. These preparations, comprising at least one active compound according to the invention alone or in combination, optionally comprise further pharmaceutically suitable auxiliaries and additives such as, for example, carrier substances, preservatives, stabilizers, emulsifiers, detergents, solvents, solubilizers, salts for regulating the osmotic pressure and buffer salts. In addition, they may comprise further therapeutically relevant active compounds, adjuvants and also regeneration-promoting substances such as, for example, growth factors or anti-inflammatory agents.

Pharmaceutically suitable materials are the compounds known to be suitable for use in the field of pharmacy and food technology and related fields, in particular those listed in relevant pharmacopeias, whose properties do not exclude them from physiological administration.

The effects caused by the compounds according to the invention also depend on their formulation. Appropriate pharmaceutical preparations should allow direct administration of the pharmaceutical into the cochlea of the mammal. Suitable administration forms of the pharmaceutical preparations according to the invention comprising at least one active compound of formula (1) or (2) can be, for example, solutions, suspensions, sprays, gels, hydrogels, lotions, emulsions, pastes, ointments or creams.

The pharmaceutical preparations according to the invention are prepared in a customary manner by processes known to the person skilled in the art, as described in relevant pharmacopeias, for example by mixing, granulation or layering methods. The

pharmaceutical preparations according to the invention may additionally be sterilized.

The invention also relates to the use of the aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention as regeneration-promoting active compounds for treating, in humans and animals, disorders associated with damaged postmitotic tissues, preferably for recovery of hearing after loss or damage of sensory hair cells in the inner ear. To this end, a person or an animal in need of such a treatment is administered a therapeutically effective amount of a regeneration-promoting preparation according to the invention comprising at least one compound of the formula (1) or (2) according to the invention. Here, the active compound or the pharmaceutical preparation according to the invention is applied directly or indirectly (including systemically), preferably locally, directly to/onto the damaged tissue structures. Administration onto or into the inner ear takes place, for example, transtympanally by injection into the middle ear, by application onto the round or oval window of the inner ear or by injection into the inner ear. Various systems for active compound administration (gel formulations, pumps) may be employed.

The compounds and pharmaceutical preparations according to the invention may be employed on their own, in combination with other compounds according to the invention or in combination with one or more active compounds relevant for the respective therapeutic indication described of the compounds according to the invention. There are no restrictions with regard to the sequence of administration. A compound according to the invention may be administered simultaneously with, before or after the other active compounds, as a

separate pharmaceutical or as a combination preparation, and by the same or by different administration routes.

The exact therapeutically effective amount for the treatment of inner ear hardness of hearing in a subject depends on various factors, *inter alia* on the extent of the tissue damage, on the height, stature, age and state of health of the patient, on the administration route and the administration form, the compound actually employed and, if appropriate, other pharmaceuticals used. Thus, at the current point in time, it is not expedient to specify the exact amount. In principle, however, repeated administration of the pharmaceutical preparations according to the invention over a period of up to 8 weeks at intervals of from one to seven days to continuous administration using "sustained release" systems provided with a mechanism for the metered sustained release of active compounds may be assumed. The amount of active compound employed should be in the range of from 0.5 µg to 1.0 mg per inner ear and administration.

Commercial utility

Regeneration biologically relevant compounds capable of inducing corresponding cell biological changes such as dedifferentiation, proliferation or terminal redifferentiation and their formulations represent a novel form of active compounds since the therapeutic concept is completely new.

Using the aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention, it was possible, for the first time, to demonstrate cell division of otic supportive cells induced by low-molecular-weight compounds, and the

resulting regeneration of sensory hair cells in the highly differentiated postmitotic tissue of the organ of Corti in mammals. Comparable methods for the *in vivo* regeneration of sensory hair cells based on pharmaceutically active compounds have hitherto not been described.

By virtue of the observed activity profiles of the compounds according to the invention, these compounds are suitable for use as pharmaceutically active compounds for the causal therapeutic treatment of sensorineural hearing loss on a regeneration biological basis. In principle, this therapeutic approach is superior to all other methods discussed to date, such as gene therapy or stem cell transplantation. In the *in vitro* and *in vivo* models examined to date, no negative effects were observed.

More details on particularly preferred embodiments and further features and advantages of the invention are evident from the following description of working examples in connection with the dependent claims, where the individual features mentioned above and still to be illustrated below are in each case claimed per se and in any combinations with one another.

The working examples are purely for illustration and do not limit the scope of the invention.

Working examples

Example A - Synthesis of 2-[1-aminoalkyl]oxazole-4-carboxylic acid amides and the corresponding 2-[1-aminoalkyl]thiazole-4-carboxylic acid amides

The synthesis of the compounds according to the invention was carried out starting with the amino acid

amides making use of relevant publications for preparing similar compounds (Videnov et al., 1996; Stanchev et al., 1999; Stankova et al., 1999; Kaiser et al; 2000).

Both the intermediates and the end products were examined for purity and identity by HPLC and mass spectrometry. In addition, the end products were characterized by NMR spectroscopy. All starting materials and reagents are commercially available.

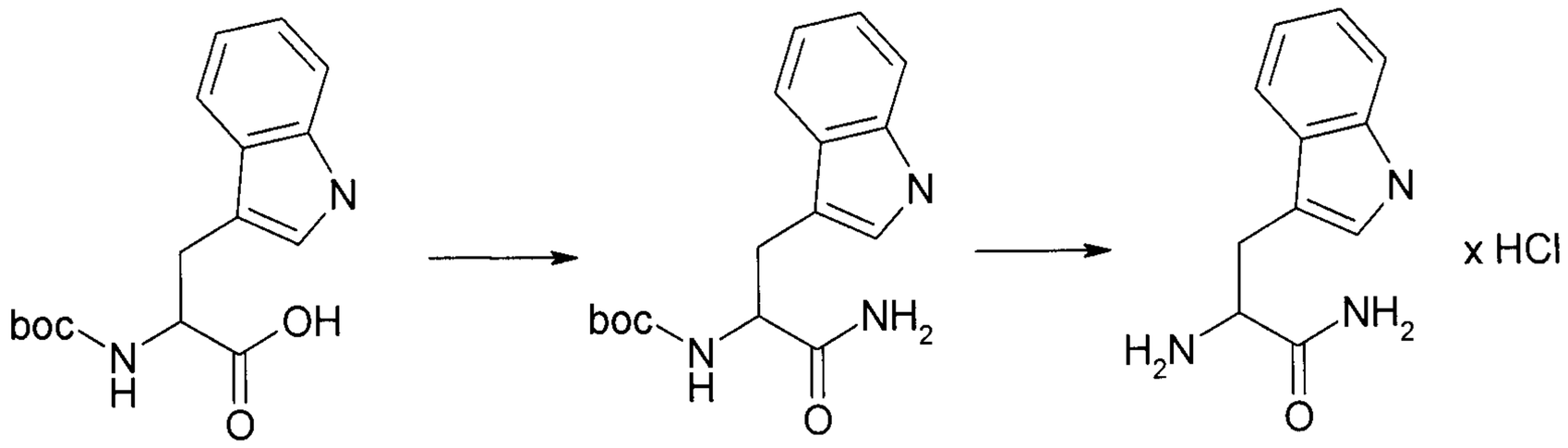
What is described hereinbelow is a synthesis route on a solid polymer phase, starting with an amino acid amide. Since a great part of the synthetic procedures are identical, no separate synthesis processes were used for oxazoles and thiazoles. At the outset, the synthesis of tryptophanamide (11) as starting material for the particularly preferred compounds (3) and (4) is shown in an exemplary manner.

An alternative synthesis route in solution was then described for the thiazoles only. However, for the experienced person skilled in the art, it is not difficult to adapt the synthesis conditions to the oxazoles.

In the descriptions of the experiments, the abbreviations X, R1 and R3, which have the same meanings as in the patent claims, were used for the variable substituents.

The syntheses described allow the preparation of the compounds in stereochemically pure form. They are obtained either in free form or as a salt, provided salt-forming groups are present.

Preparation of D-tryptophanamide (11)

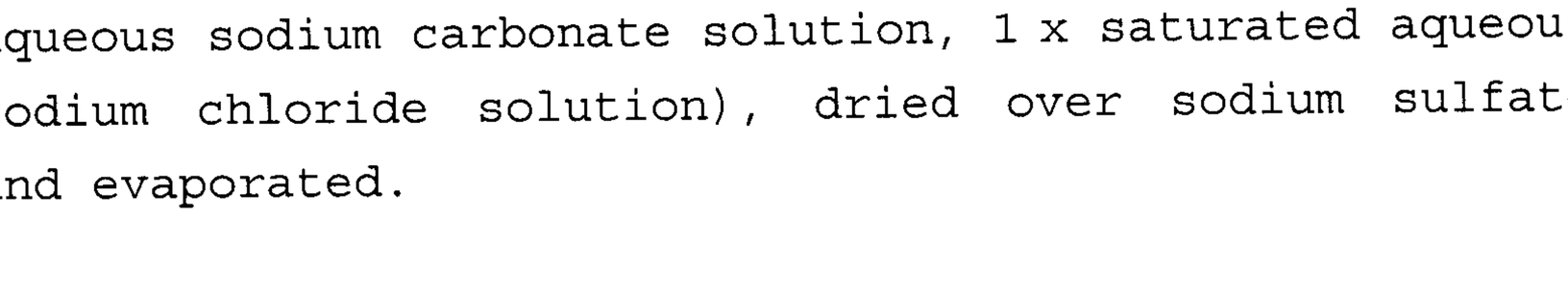


(9) **(10)** **(11)**

N-Boc-D-Tryptophan (9) (1 equiv.; 2.75 mmol; 1.00 g), hydroxybenzotriazole ammonium salt (2 equiv.; 5.50 mmol; 924 mg) and diisopropylcarbodiimide (DIC) (1.1 equiv.; 3.03 mmol; 382 mg; 454 µl) were dissolved in dimethylformamide (DMF) which had been dried over molecular sieves, and the solution was stirred at room temperature for 18 h. The solvent was then removed on a rotary evaporator under reduced pressure, and the residue was taken up in ethyl acetate. Insoluble residues were filtered off, and the organic phase was then washed (1 x 1 M $KHSO_4$ solution, 2 x saturated aqueous sodium carbonate solution, 1 x saturated aqueous sodium chloride solution), dried over sodium sulfate and evaporated.

To remove the Boc protective group, the crude product (10) was taken up in a solution of 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) and stirred at room temperature for 3 h. After evaporation of the solvents, product (11) was recrystallized.

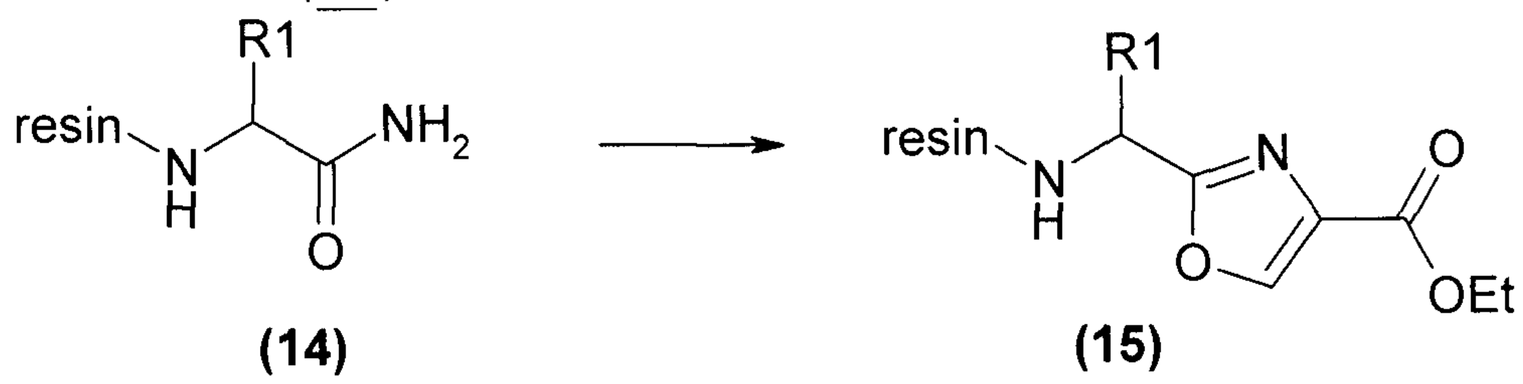
Loading of chloro-(2'-chloro)trityl polystyrene resin (12) with amino acid amide (13)



(12) **(13)** **(14)**

Chloro-(2'-chloro)trityl polystyrene resin (12) (Rapp Polymere H10033; loading 1.31 mmol/g) (1 equiv.; 655 µmol; 500 mg) was washed twice with DMF (over molecular sieve), a solution of the amino acid amide hydrochloride (13) (2 equiv.; 1.31 mmol) and diisopropylethylamine (DIPEA) (5 equiv.; 3.28 mmol; 424 mg) in DMF was added and the mixture was shaken at room temperature for 18 h. To cap the resin, methanol (MeOH) was then added to the suspension and the mixture was shaken at room temperature for another 15 min. The reaction solution was filtered off with suction, the loaded resin (14) was washed (5 x DMF, 3 x each MeOH, tetrahydrofuran (THF), diethyl ether) and dried under oil pump vacuum.

Cyclization of the immobilized amino acid amide (14) to the oxazole (15)

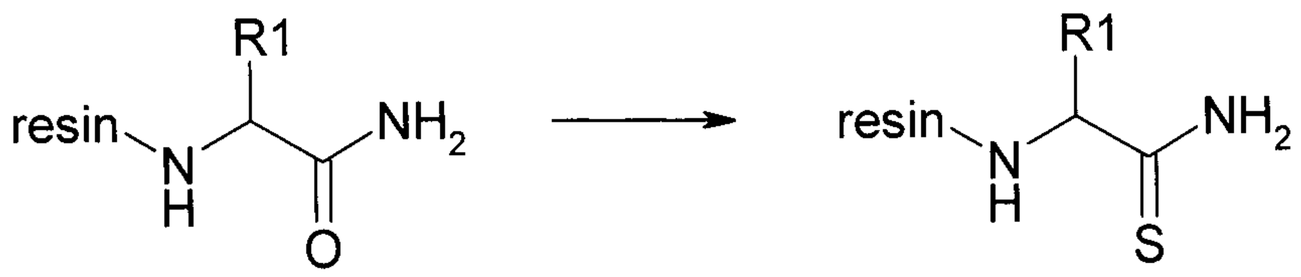


Ethyl bromopyruvate (5 equiv.; 3.28 mmol; 640 mg; 412 µl) and N,N-dimethylaniline (10 equiv.; 6.55 mmol; 794 mg; 832 µl) were dissolved in dioxane (5 ml) and added to the amino acid amide resin (14) (1 equiv.; 655 µmol; 500 mg). The suspension was shaken at room temperature for 16 h and then warmed at 60°C for 2 h. The reaction solution was filtered off with suction and the resin was washed with dry solvents (5 x DMF, 5 x DCM).

A solution, cooled to -20°C, of pyridine (10 equiv.; 6.55 mmol; 520 mg; 530 µl) in dry DCM (5 ml) was mixed with trifluoroacetic anhydride (5 equiv.; 3.28 mmol; 688 mg; 456 µl) and added to the washed resin. After

30 min at -20°C, the suspension was warmed to room temperature and shaken at room temperature for a further 2 h. The reaction solution was filtered off with suction and the resin (15) was washed (3 x each DMF, MeOH, THF, DCM, diethyl ether) and dried under reduced pressure in a desiccator.

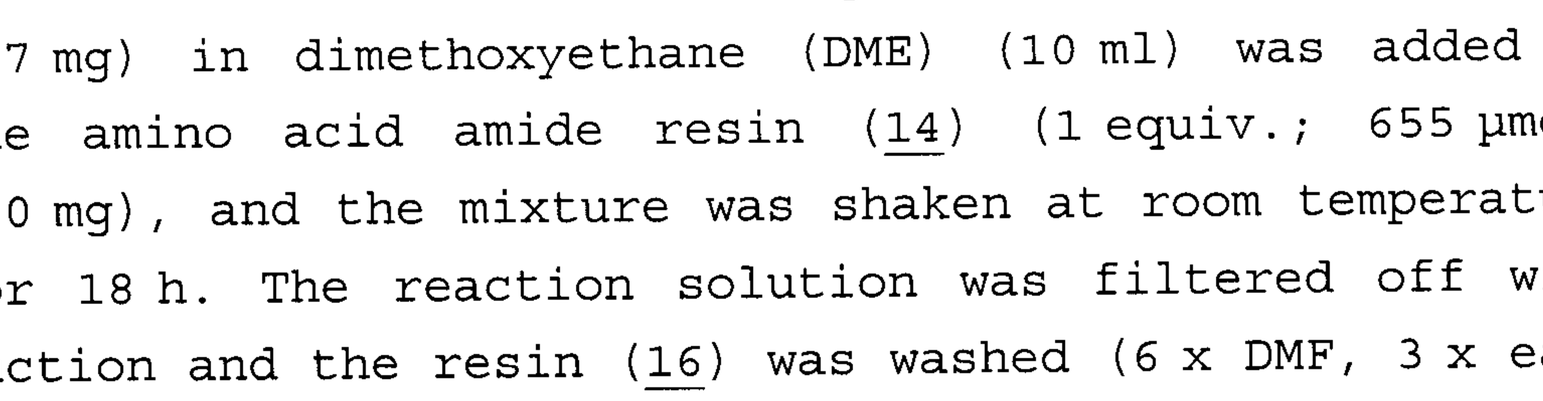
Preparation of immobilized thioamides (16) with Lawesson's reagent



(14) **(16)**

A solution of Lawesson's reagent (3 equiv.; 1.97 mmol; 797 mg) in dimethoxyethane (DME) (10 ml) was added to the amino acid amide resin (14) (1 equiv.; 655 µmol; 500 mg), and the mixture was shaken at room temperature for 18 h. The reaction solution was filtered off with suction and the resin (16) was washed (6 x DMF, 3 x each MeOH, THF, DCM, diethyl ether) and dried under reduced pressure in a desiccator.

Cyclization of the immobilized thioamide (16) to thiazole (17)



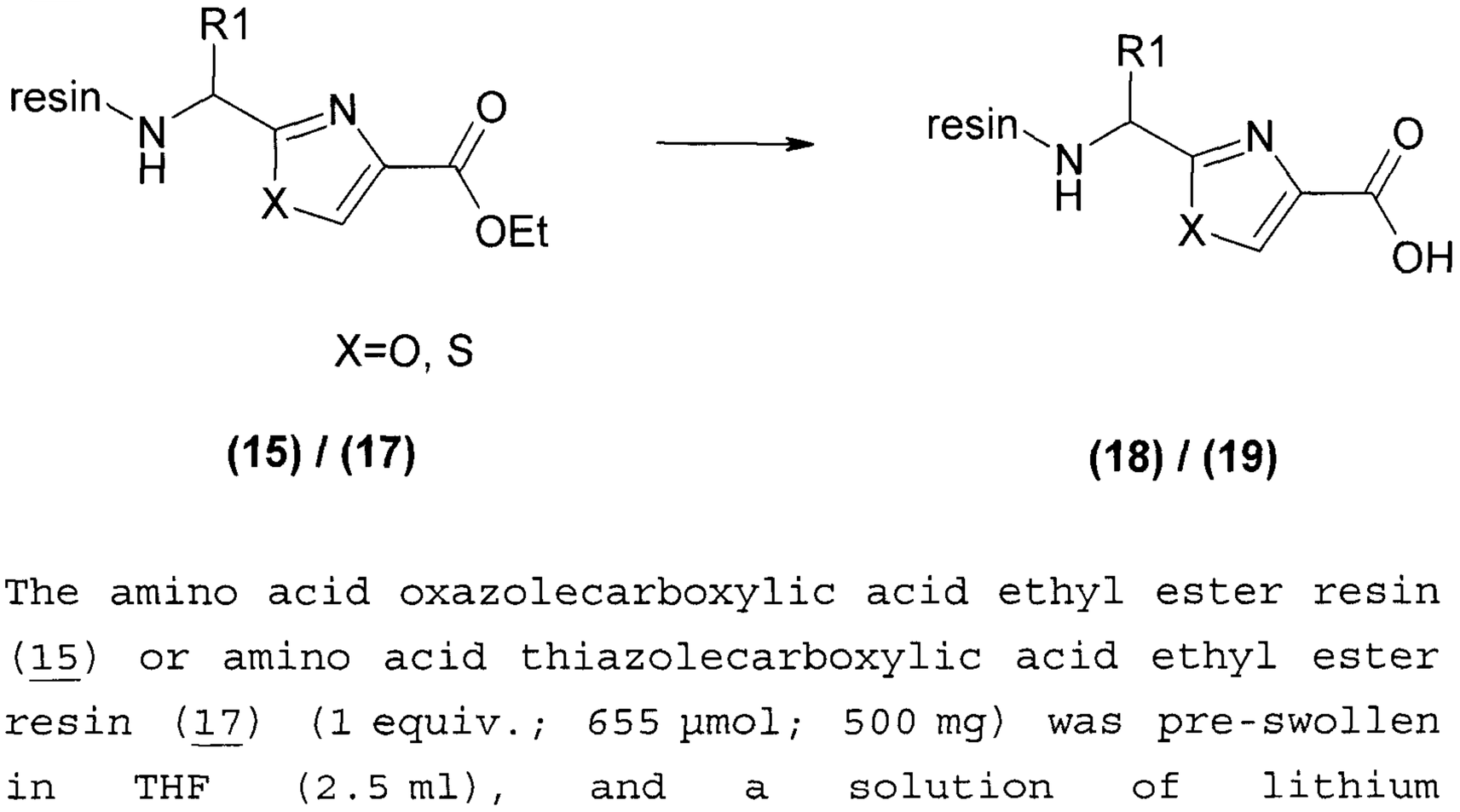
(16) **(17)**

Ethyl bromopyruvate (5 equiv.; 3.28 mmol; 640 mg; 412 µl) and N,N-dimethylaniline (5 equiv.; 3.28 mmol; 397 mg;

416 µl) were dissolved in DMF (4 ml) and added to the amino acid thioamide resin (16) (1 equiv.; 655 µmol; 500 mg). The suspension was shaken at room temperature for 16 h. The reaction solution was filtered off with suction and the resin (17) was washed (3 x each DMF, MeOH, THF, DCM, diethyl ether) and dried under reduced pressure in a desiccator.

After a test cleavage using 5% TFA in DCM at room temperature for 1 h, identity and purity of reaction product (17) were checked by chromatography and mass spectrometry.

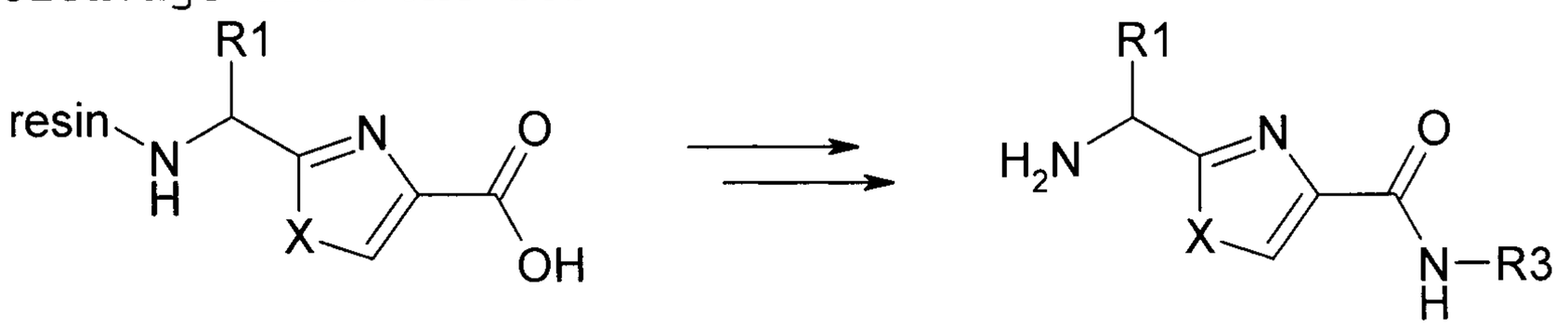
Hydrolysis of the immobilized ethyl esters (15) and (17)



(15) / (17) **(18) / (19)**

The amino acid oxazolecarboxylic acid ethyl ester resin (15) or amino acid thiazolecarboxylic acid ethyl ester resin (17) (1 equiv.; 655 µmol; 500 mg) was pre-swollen in THF (2.5 ml), and a solution of lithium hydroxide monohydrate (5 equiv.; 3.28 mmol; 137 mg) in water (1.25 ml) and MeOH (1.25 ml) was added. After 3 h at room temperature, the reaction solution was filtered off with suction, the resin (18) or (19) was washed (3 x each water, water / DMF 1:1, DMF, dioxane, THF, DCM, diethyl ether) and dried under reduced pressure in a desiccator.

Amidation of the carboxylic acids (18) and (19) and cleavage from the resin



X= O, S

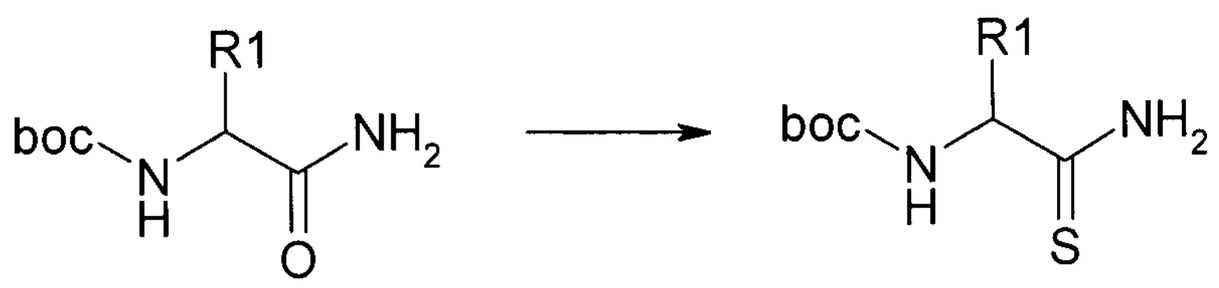
(18) / (19) **(20) / (21)**

A solution of 1-hydroxybenzotriazole (HOBt) (5 equiv.; 3.28 mmol; 443 mg) and DIC (5 equiv.; 3.28 mmol; 413 mg) in dry DMF (5 ml) was added to the amino acid oxazolecarboxylic acid resin (18) or amino acid thiazolecarboxylic acid resin (19) (1 equiv.; 655 µmol; 500 mg). After 30 min of shaking at room temperature, the R3-amine (10 equiv.; 6.55 mmol) was added and the suspension was shaken at room temperature for a further 16 h. The reaction solution was filtered off with suction and the resin was then washed (3 x each DMF, MeOH, THF, DCM, diethyl ether) and sucked dry in a stream of air.

The product (20) or (21) was then cleaved from the resin at room temperature using a solution of 5% TFA in DCM for 1 h.

After evaporation of the cleavage solutions, the crude product was lyophilized from tBuOH / water 4:1 and chromatographed on silica gel using a DCM/MeOH gradient.

Preparation of the thioamides (23) with Lawesson's reagent in solution

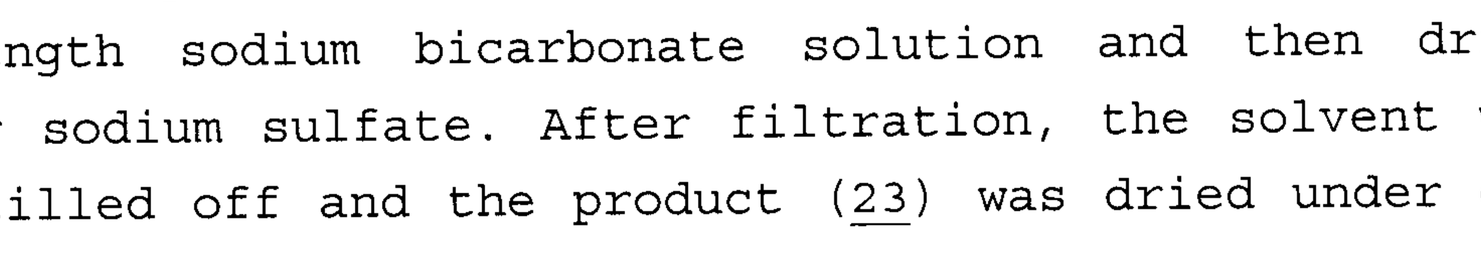


(22) **(23)**

With stirring, Lawesson's reagent (0.75 equiv.; 750 µmol; 305 mg) was added to a solution of the Boc-amino acid amide (22) (1 equiv.; 1.00 mmol) in dry DME (7.5 ml). The reaction solution was stirred at room temperature for 16 h and the solvent was then removed by distillation on a rotary evaporator under reduced pressure. The residue was taken up in ethyl acetate (30 ml) and stirred vigorously with 10% strength sodium bicarbonate solution (15 ml) for 30 min. After separation of the phases, the aqueous phase was extracted twice with ethyl acetate. The combined organic phases were washed three times with 10% strength sodium bicarbonate solution and then dried over sodium sulfate. After filtration, the solvent was distilled off and the product (23) was dried under oil pump vacuum.

The crude products can be recrystallized from ethyl acetate or ethyl acetate / petroleum ether or purified by flash chromatography on silica gel.

Cyclization of thioamides (23) to thiazole (24) in solution



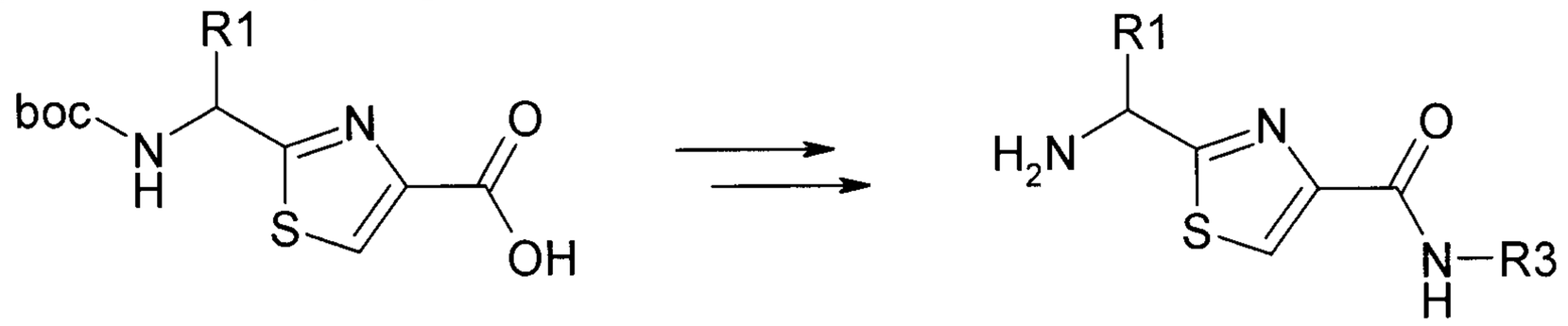
(23) **(24)**

Calcium carbonate (2 equiv.; 1.60 mmol; 161 mg) was added to a solution of the Boc-amino acid thioamide (23) (1 equiv.; 0.80 mmol) in anhydrous ethanol (6 ml),

and the suspension was stirred at room temperature for 10 min. Bromopyruvic acid (1.5 equiv.; 1.20 mmol; 201 mg) was then added. After 4 h of stirring at room temperature, the reaction mixture was filtered and the residue was washed with ethanol. The combined filtrates were concentrated on a rotary evaporator under reduced pressure, and the residue was taken up in ethyl acetate (10 ml) and extracted three times with 10 ml of 5% strength potassium bisulfate solution and once with saturated sodium chloride solution. The organic phase was then dried over sodium sulfate. After filtration, the solvent was distilled off and the product (24) was dried under oil pump vacuum.

The crude products were purified by flash chromatography on silica gel.

Amidation of thiazole (24) and removal of the Boc protective group in solution



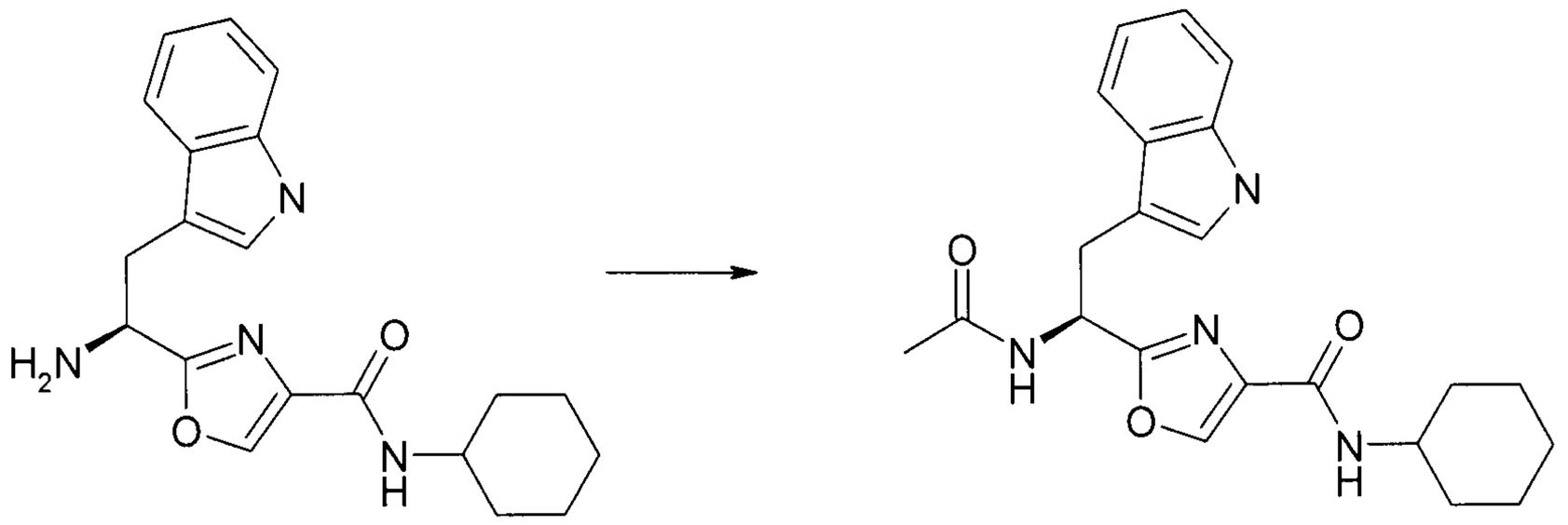
(24) **(25)**

(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (1.05 equiv.; 0.84 mmol; 437 mg) and triethylamine (TEA) (3 equiv.; 2.40 mmol; 242 mg; 330 µl) were added to the Boc-amino acid thiazolecarboxylic acid (24) (1 equiv.; 0.80 mmol) in THF, and the mixture was stirred at room temperature for 30 min. The R3-amine (1.3 equiv.; 1.04 mmol) was then added, and the mixture was stirred at room temperature for 18 h.

The reaction solution was evaporated and the crude product was chromatographed on silica gel using a DCM/MeOH gradient.

To remove the Boc protective group, the amide was stirred in 25% TFA in DCM at room temperature for 1 h and then evaporated. Repeatedly, heptane was added to the crude product and removed by distillation again on a rotary evaporator under reduced pressure. The product (25) was then lyophilized from tBuOH / water 4:1.

N-Acetylation of N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3)



(3) (4)

Acetic anhydride (5 equiv.; 140 µmol; 14 mg; 13 µl) and TEA (2 equiv.; 56 µmol; 6 mg; 8 µl) were added to the aminoalkyloxazolecarboxylic acid amide (3) (1 equiv.; 28 µmol; 10 mg) in DCM (0.5 ml), and the mixture was stirred at room temperature for 18 h. The reaction solution was then evaporated and the crude product (4) was lyophilized from tBuOH / water 4:1.

Example B - Demonstration of the regenerative properties in the *in vitro* cell culture assay

First, starting with stem cells with otic development potential isolated from the postnatal organ of Corti of the mouse, typical cellular cluster "spheres" were

grown in a suspension culture. Under culture conditions optimized compared to known processes (Oshima et al., 2007; Senn et al., 2007) in a *DMEM / F12* medium (Dulbecco's Modified Eagle Medium) supplemented with *B27* and *N2*, about 1600 solid spheres per organ of Corti could be generated from the stem cell culture with added *FGF* (fibroblast growth factor) and *IGF* (insulin-like growth factor) (Figure 1C).

By labelling on the protein level (Figure 1A) and demonstrating a plurality of stem cell markers on the mRNA level (Figure 1B), it was shown that the otospheres formed under these culture conditions are in a dedifferentiated state which corresponds to an earlier state during ontogenesis in the organ of Corti. In addition, it was possible to demonstrate cell divisions in the otospheres.

In situ, supportive cells are the potential precursors for a regeneration of sensory hair cells and therefore the actual target cells for the induction of sensory hair cell regeneration. For this purpose, it was necessary to establish *in vitro* a joint culture of hair- and supportive cell-like cells constituting a relatively good representation of the cellular composition of the organ of Corti.

To this end, the cultivated otospheres were differentiated in a second step under adherent culture conditions on an ornithine / fibronectine surface to give monolayer epithelium islands relatively similar to the original sensory epithelium.

By immunocytochemical detection, it was possible to identify, on the protein level, in each case three suitable markers for supportive cells and sensory hair cells. Figure 2 shows a comparison of the situation in

the native organ (*in vivo*) and in the differentiated epithelium islands of the cell culture (*in vitro*) using these markers.

These preliminary studies carried out by prior art methods and without addition of the compounds according to the invention showed that the cultivated otic epithelium islands are suitable as a cellular base for demonstrating the regenerative potential of the compounds according to the invention for supportive cell-like cells of the inner ear. Changes of the protein expression of markers for supportive cells, hair cells and stem cells can be use to demonstrate the effects mediated by the substances administered.

For screening, firstly the proportion of *Sox2*-positive cells in the *in vitro* culture was determined. *Sox2* is a stem cell marker, which is also expressed during ontogenesis of the organ of Corti. It is thought to have an important role in the pluripotency of embryonal stem cells and during induction of pluripotent stem cells from differentiated cells (Takahashi and Yamanaka, 2006). Accordingly, *Sox2* is an important marker, in particular in the context of an induced dedifferentiation/reprogramming of cells.

By adding the compound according to the invention N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) to the cell culture, the proportion of *Sox2*-expressing cells could be more than doubled compared with the differentiation control (Figure 3).

If the expression of stem cell markers induced by the compounds is in the end also associated with an increased proliferation of the cells in culture was checked by quantification of the *BrdU*-positive cells.

Here, during substance administration the culture was incubated for 5 h with the thymidine analog *BrdU*. During the S phase of the cell cycle, cell division results in incorporation of *BrdU*. If one of the substances stimulates cell division, this can be detected and visualized with an antibody directed against *BrdU*.

By adding the compound according to the invention N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) to the cell culture, it was possible to induce proliferation in 4.8% of the cells differentiated beforehand (Figure 4).

Both with regard to *Sox2* expression and *BrdU* incorporation, the compound according to the invention 2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid N-cyclohexylamide (3) achieved significant positive results. In this manner, it was shown that the compound is capable of inducing dedifferentiation and subsequent proliferation of otic supportive cells.

In a dose/activity analysis, the optimum concentration range in which this compound unfolds its maximum regeneration biological effect in the cell culture was determined.

To this end, the incorporation of *BrdU* in the stem cell-based *in vitro* cell culture assay was determined at 4 concentrations between 0.3 µM and 10 µM in the culture medium. It was found that with the concentration of 0.3 µM, no significant effect was achieved. However, at a concentration of 1 µM, the mean was already elevated. From a concentration of 3 µM, the saturation level of about 9% *BrdU*-positive cells is already reached (Figure 5).

Example C - Demonstration of the regenerative properties in the *in vitro* organ culture model

To confirm the effects observed in the cell culture assay in the native organ, i.e. *in situ* in the complex cellular structure of the organ of Corti, use was made of a form of organ culture where the tissue to be cultivated, in the present case the entire inner ear of the mouse, was located in a rotating cylinder filled with culture medium (Hahn et al., 2008). Before the start of the experiment, the cochlea was opened basally and apically in the region of the *scala tympani*. In this manner, it was possible to minimize the effect of gravity and at the same time to achieve optimum gas and nutrient exchange between the tissue of the organ of Corti and the culture medium. Under these conditions, the explantate could be kept in culture for longer compared to a stationary culture.

To demonstrate that, as a result of the administration of the compounds according to the invention, the supportive cells remaining in the organ of Corti after loss of sensory hair cells dedifferentiate to precursor cells and then proliferate, a situation analogous to a hard of hearing ear was established. After administration of the ototoxic aminoglycoside antibiotic neomycin (1 mM), $^2/_3$ of all sensory hair cells suffered cell death within 24 hours. Only in the apical regions of the cochlea, some sensory hair cells survived, the number of which declined further during the course of the experiment as a result of the initial damage. After removal of the neomycin from the medium, the remaining supportive cells could be cultivated further.

BrdU was then added to the culture medium simultaneously with the compounds according to the

invention (5 µM), and after 4 days the proliferation was quantified by *BrdU* incorporation.

After addition of the compound N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) according to the invention to the organ culture, *BrdU* incorporation could be demonstrated in the furthest laterally orientated Deiter's cells (Figure 6B). This was also the case for individual inner phalangeal cells and border cells (Figure 6C) which are associated with the inner sensory hair cell and can be considered to be potential precursor cells for their regeneration. In control experiments without substance administration, there was no spontaneous incorporation of *BrdU* into Deiter's cells, inner phalangeal cells and inner border cells (Figure 6A).

The different morphology of the *BrdU*-positive nuclei indicated various stages of mitosis up to completed cell division. Chromatin condensation could be observed in various nuclei (Figures 6D, E). At the same time, *BrdU*-positive nuclei were found in immediate relative proximity of one another (Figures 6E, F).

This means that, owing to the action of the compound N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) according to the invention, new cells in the sense of a regeneration by cell division of supportive cells have been formed in the sensory epithelium of the organ of Corti.

Example D - Demonstration of the regenerative properties after *in vivo* administration

The regeneration biological effect of the compounds according to the invention was likewise demonstrated in the *in vivo* model of the adult guinea pig. Acute

acoustic damage resulting in a loss of sensory hair cells of the organ of Corti, in particular in the region of the outer sensory hair cells, was caused by impulse noise. The compound N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) according to the invention was then continuously administered locally from a miniosmotic pump (Alzet®), subcutaneously implanted, via choleostomy directly into the *scala tympani* of the cochlea. The dosage of the substance in the pump was correspondingly higher at 200 µM, taking into account an expected dilution effect in the perilymph.

Administration was over a period of 6 weeks, with a subsequent waiting interval of 2 weeks. In parallel, *BrdU* was administered via the drinking water or the miniosmotic pump to label proliferating cells in the organ of Corti.

After removal of the organ of Corti, immunohistochemical triple labelling with *BrdU* (cell division), *myosin VI* (sensory hair cells) and *DAPI* (staining of the nuclei) (Figure 7) and also with *Sox2* (pluripotent supportive cells) was carried out.

BrdU-labelled supportive cells and sensory hair cells were preferably demonstrated in the regions of the organ of Corti damaged by acoustic trauma. In control organs of the opposite side with/without acoustic damage and without substance administration, no *BrdU*-labelled sensory hair cells and, altogether, in accordance with the known findings on spontaneous proliferation, only few *BrdU*-labelled cells were found.

The *in vivo* findings show unambiguously that re-generation of sensory hair cells on the basis of cell divisions can be induced even in the adult animal by

administration of the compound N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) according to the invention. This underlines the surprisingly great potential of the compounds according to the invention as active compounds for the causal treatment of inner ear hardness of hearing by regeneration of sensory hair cells.

Example E -Toxicity

In the cell culture, organ culture and *in vivo* studies, no indications of toxic effects of the aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides according to the invention were found in the concentration range examined from 0.3 µM to 200 µM.

Figures

Figure 1. Isolation and cultivation of stem cells from the organ of Corti of the mouse.

(A) Stem cells from the inner ear can be obtained by individualizing the cells of the postnatal organ of Corti, isolating the stem cells still present and cultivating them under selective conditions in a suspension culture. Under these conditions, spheres are formed which express the stem cell marker *Sox2* (A´) which is thought to have a decisive role in the pluripotency of embryonal stem cells and in the induction of pluripotent stem cells from differentiated cells (Takahashi and Yamanaka, 2006). Moreover, the nuclei in the spheres incorporate *BrdU* (A´´) which indicates the proliferation of the cells.

(B) Several stem cell markers which can also be detected during ontogenesis in the organ of Corti are expressed in the spheres. As positive control (pos),

the mRNA from an organ of Corti of the embryonal development day 14.5 was applied. Standardized to the household gene *GAPDH*, it was possible to show that *Jag1*, *Nestin*, *Sox2* and *Nanog* are expressed both in the inner ear being formed and in the stem cells isolated therefrom.

(C) Using optimized methods, it was possible to double the proportion of spheres formed from the primary culture compared to the currently published literature (Oshima et al., 2007; Senn et al., 2007).

Figure 2. *In vivo* and *in vitro* protein expressions profile of hair and supportive cell markers.

(A) $p27^{Kip1}$ is expressed in all supportive cells of the organ of Corti. Neither inner nor outer sensory hair cells are $p27^{Kip1}$-positive. It was possible to demonstrate $p27^{Kip1}$ expression within the epithelial islands (A´).

(B) *GFAP* labelling of supportive cells in the organ of Corti immediately below the nuclei of Deiter's cells and in the region of the inner phalangeal cells. The pattern of the filaments was also found *in vitro* (B´).

(C) *E-Cadherin* labels all supportive cells orientated laterally to the pillar cells. *E-Cadherin* is also located in the mebranes of the epithelial islands (C´).

In vivo, sensory hair cell markers such as *myosin VIIA* (D), *myosin VI* (E) and *calretinin* (F) reliably label inner and outer sensory hair cells. All markers can also be detected in individual cells in the *in vitro* culture (D´, E´, F´).

In the native organ, the asterisks in each case mark the 3 outer sensory hair cells and the inner sensory hair cell (always on the right-hand side).

Scale: 20 µm in the pictures of the naive organ of Corti (A-F),
10 µm in the cell culture (A'-F').

Figure 3. Relative number of cells expressing *Sox2* in the screening assay.

It was examined which of the low-molecular-weight compounds were capable of inducing an increase in the proportion of *Sox2*-positive cells (as marker for the dedifferentiation of cells, standardized to the level of the differentiation control).

Seven compounds differ significantly from the control ($p < 0.05$, $n = 10$).

Figure 4. Relative number of *BrdU*-positive cells in the screening assay.

By detection of *BrdU*, it is possible to quantify how many cells have entered the S phase of the cell cycle within a certain period.

During screening, the cells were incubated with *BrdU* for five hours.

What is shown is the proportion of cells in the total population stimulated to proliferate by substance administration.

Eight compounds were able to induce significant proliferation ($p < 0.05$, $n = 10$).

Figure 5. Dose/activity analysis for N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) (substance 33).

In the adhesion culture, the dose-dependency of the induced *BrdU* effect was checked. When the substance concentration in the culture medium was increased from 0.3 µM to 3 µM, the *BrdU* incorporation increased to a significantly higher level ($p < 0.05$, $n = 10$). At higher concentrations, the *BrdU* incorporation could not be increased any further. The control, a population of cells from the *in vitro* culture with a comparable amount of DMSO without substance administration did not mediate any effect.

Figure 6. Incorporation of *BrdU* induced by N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) (substance 33) in the organ culture model.

The extent of the induced proliferation of supportive cells was demonstrated by incorporation of *BrdU*.

In the control without substance administration, no incorporation of *BrdU* was observed in the Deiter's cells, the inner phalangeal cells and the border cells (A). After administration of the compound (3) according to the invention (substance 33, 5 µM), *BrdU* was incorporated into laterally orientated Deiter's cells (B) and also into individual inner phalangeal cells and inner border cells (C).

The *BrdU*-positive nuclei were at different stages of mitosis or had completed cell division (D, E, F).

Scale: 20 µm.

Figure 7. Triple labelling after intracochlear administration of N-cyclohexyl-2-[1-amino-2-(1H-indol-3-yl)ethyl]oxazole-4-carboxylic acid amide (3) *in vivo*.

In the *in vivo* experiment with adult guinea pigs, the regenerative effect of the compounds according to the invention was demonstrated under reality-like conditions (administration over a period of 6 weeks using an Alzet® pump (200 µM) in parallel with *BrdU* (100 mg/ml), removal of the organ and staining after 8.5 weeks).

What is shown are inner sensory hair cells labelled with *myosin VI* (marker for sensory hair cells) and *BrdU* (marker for cell division). The cell nucleus is labelled with DAPI (nucleus staining).

Labelling with *BrdU* shows regeneration based on cell division.

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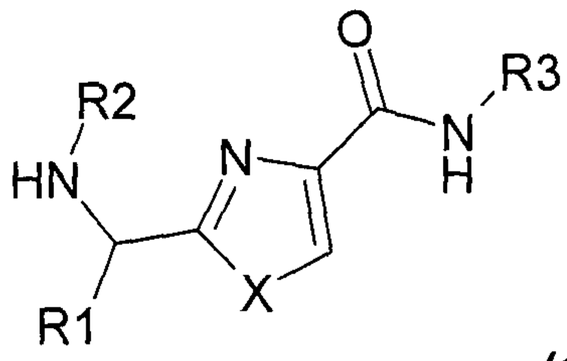
Claims

1. Aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides of the formulae



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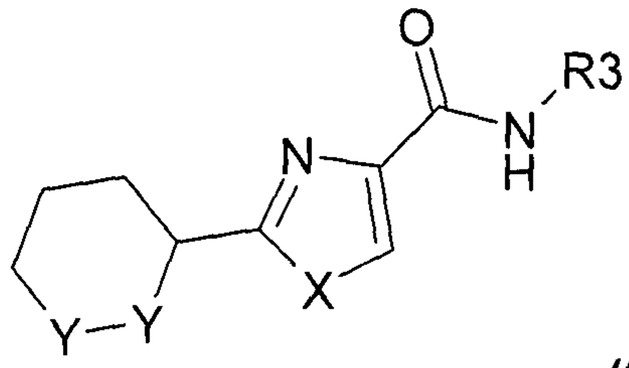


(2)

where

X represents O or S,

Y represents C or N, where the two atoms must be different from one another,

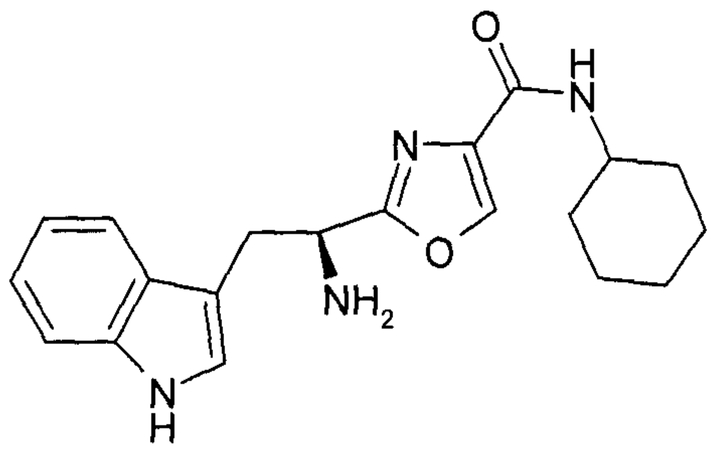
R2 represents hydrogen or acyl and

R1 and R3, which may be identical or different, represent a substituent selected from the groups below: branched or straight-chain, substituted or unsubstituted alkyl groups, alkylcycloalkyl groups, alkylaryl groups, cycloalkyl groups, cycloalkylaryl groups, aryl groups and arylcycloalkyl groups which optionally contain heteroatoms, where R1 preferably represents (1H-indol-3-yl)-ethyl and R3 preferably represents cyclohexyl,

or

a pharmaceutically acceptable salt, a stereoisomer, a stereoisomer mixture, a tautomer or a prodrug compound, preferably a prodrug ester or a prodrug peptide, thereof.

2. The compound as claimed in claim 1, selected from the group consisting of



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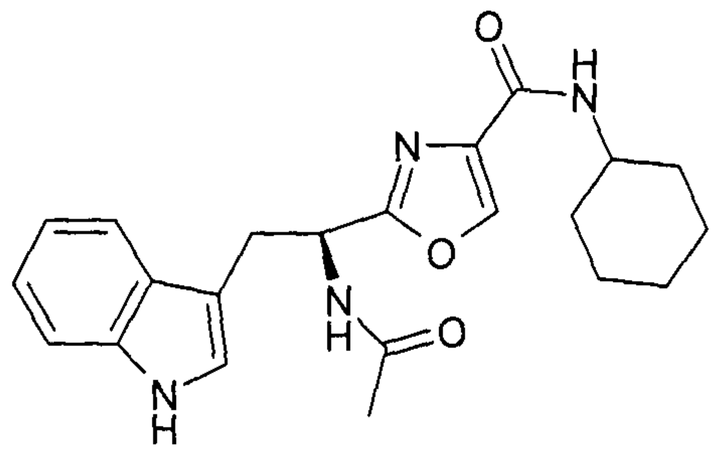

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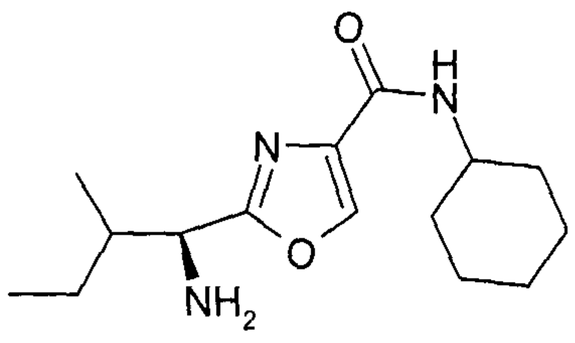

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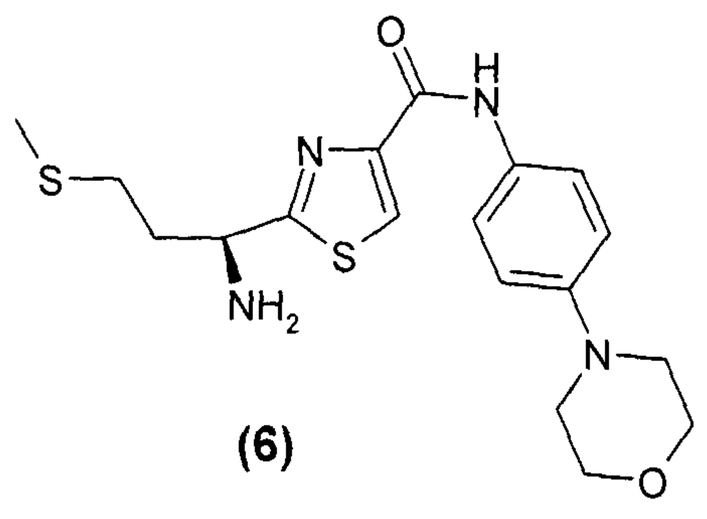

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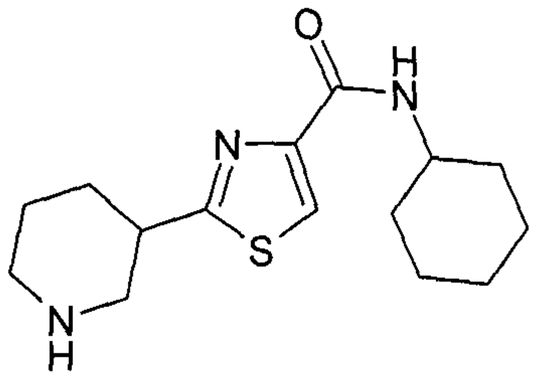

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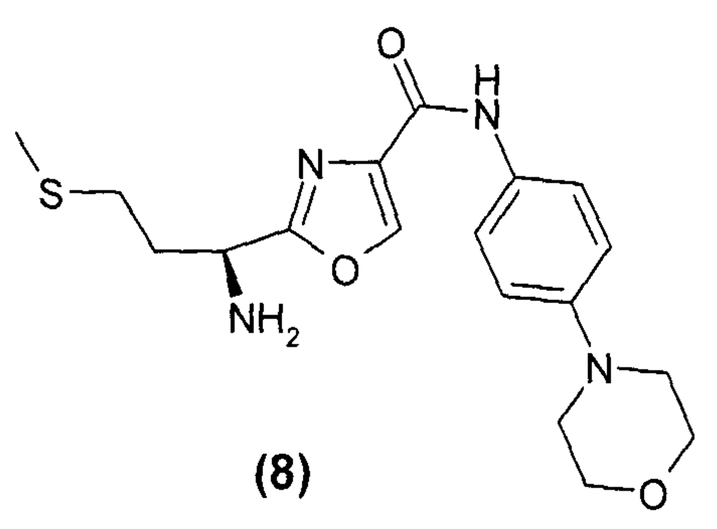

(8)

or

a pharmaceutically acceptable salt, a stereoisomer, a stereoisomer mixture, a tautomer or a prodrug compound, preferably a prodrug ester or a prodrug peptide, thereof.

3. The compound as claimed in claim 1 or 2 for use for any medical purpose, preferably as medicament for various indications in human and veterinary medicine.

4. The use of a compound as claimed in claim 1 or 2 as regeneration-promoting active compound, characterized in that, on action of a therapeutically effective amount on the damaged tissue of highly

specialized organs and tissues, preferably the brain, the heart, the skeletal muscles and particularly preferably of sensory epithelia, the endogenous *in situ* regeneration of the normally postmitotic tissue is stimulated by regeneration biological effects such as dedifferentiation, proliferation and subsequent redifferentiation of terminally differentiated cells.

5. The use of a compound as claimed in claim 1 or 2 as regeneration-promoting active compound for regenerating the otic sensory epithelia of mammals, characterized in that, on action of a therapeutically effective amount, the endogenous *in situ* regeneration of damaged and lost sensory hair cells in the organ of Corti is stimulated by regeneration biological effects such as dedifferentiation, proliferation and subsequent redifferentiation of supportive cells in the inner ear.

6. A process for preparing the aminoalkyloxazole- and aminoalkylthiazolecarboxylic acid amides as claimed in claim 1 or 2, characterized in that the cyclization to the oxazole or thiazole and their subsequent amidation are carried out starting with a natural or unnatural amino acid to be amidated on a solid phase or, using Boc protective group chemistry, in solution.

7. A pharmaceutical preparation, comprising a therapeutically effective amount of at least one aminoalkyloxazole- or aminoalkylthiazolecarboxylic acide amide as claimed in claim 1 or 2 alone or in combination, optionally further regeneration-promoting active compounds or active compounds which are relevant for the therapy in another way, and also further pharmaceutically suitable ingredients such as, for example, carrier substances, auxiliaries and additives, detergents, adjuvants, and others.

8. A pharmaceutical preparation as claimed in claim 7, characterized in that it is present in a formulation suitable for direct (local) or indirect (including systemic) administration to the damaged tissue or the cochlea of the mammal, preferably as a solution, suspension, spray, gel, hydrogel, lotion, emulsion, paste, ointment or creme.

9. A process for preparing a pharmaceutical preparation as claimed in claims 7 and 8, characterized in that the components are mixed and/or dissolved and/or associated with a physical or biological carrier.

10. The use of a compound as claimed in claim 1 or 2 or a pharmaceutical preparation as claimed in any of claims 7 to 9 for preparing a pharmaceutical for the causal treatment of mammalian disorders associated with damaged postmitotic tissues on the basis of regeneration biology by direct or indirect administration of a therapeutically effective amount to the damaged tissue structures.

11. The use of a compound as claimed in claim 1 or 2 or a pharmaceutical preparation as claimed in any of claims 7 to 9 for preparing a pharmaceutical for the causal treatment of inner ear hardness of hearing and restoration of hearing in mammals after damage and loss of the sensory hair cells in the organ of Corti on the basis of regeneration biology by direct or indirect administration of a therapeutically effective amount to the damaged tissue structures in the cochlea.

12. A method for the causal treatment of disorders of humans and animals associated with damaged postmitotic tissues on the basis of regeneration biology, characterized in that a therapeutically effective

amount of a compound as claimed in claim 1 or 2 or a pharmaceutical preparation as claimed in any of claims 7 to 9 is applied directly, preferably locally, to the damaged tissue structure or administered indirectly, preferably systemically.

13. A method for the causal treatment of inner ear hardness of hearing and for restoring hearing of humans and animals after damage and loss of the sensory hair cells in the organ of Corti on the basis of regeneration biology, characterized in that a therapeutically effective amount of a compound as claimed in claim 1 or 2 or a pharmaceutical preparation as claimed in any of claims 7 to 9 is administered directly or indirectly to the damaged tissue structures in the cochlea, preferably transtympanally by injection into the middle ear, by application to the round or oval window of the inner ear or by injection into the inner ear.

Application number / Numéro de demande: EP20110005O2

Figures: 1-2-6-7-

Pages:

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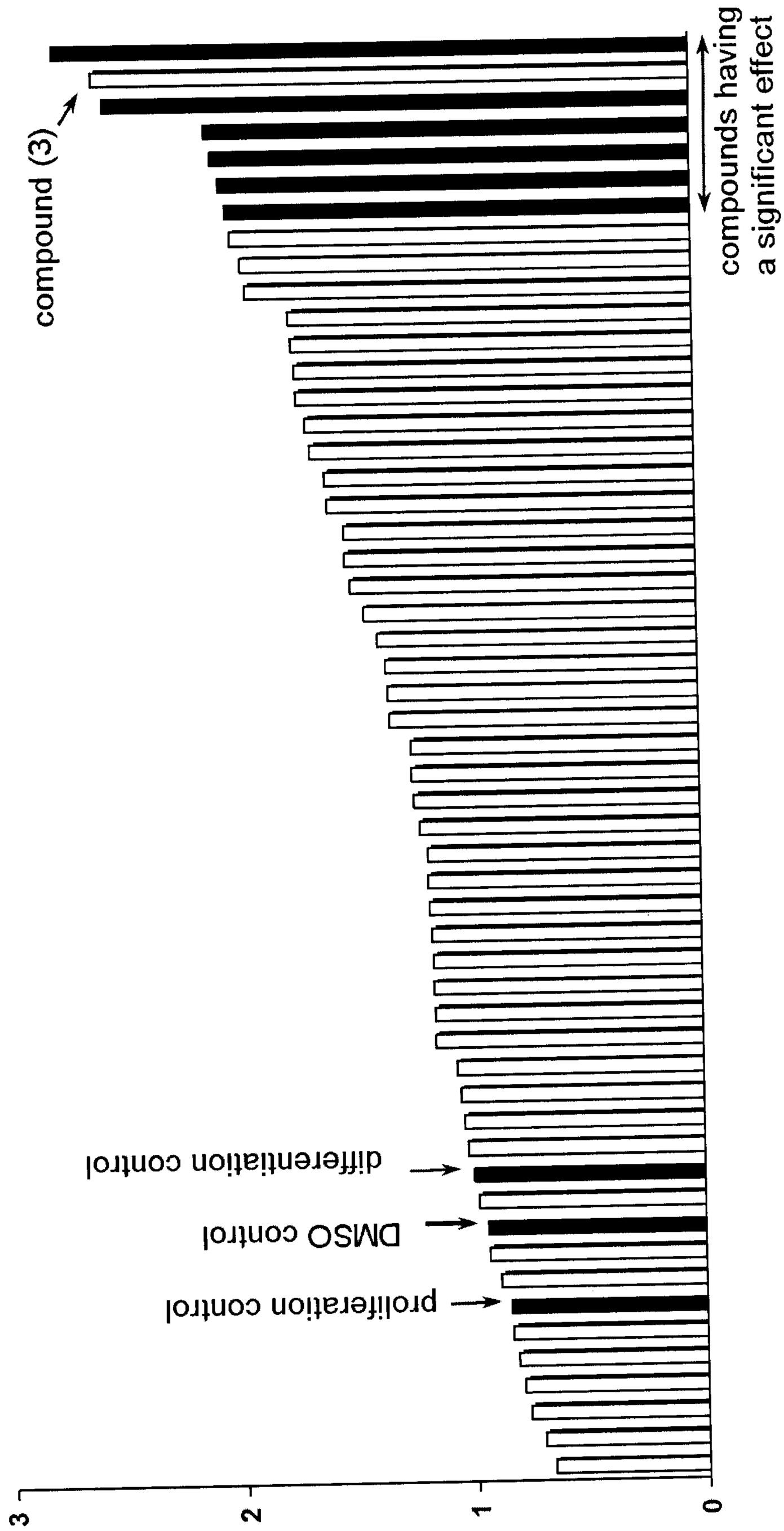

compound (3)
compounds having a significant effect
Substance-stimulated relative Sox2 expression
differentiation control
DMSO control
proliferation control
Sox2-positive cells [%]
0
1
2
3

Figure 3

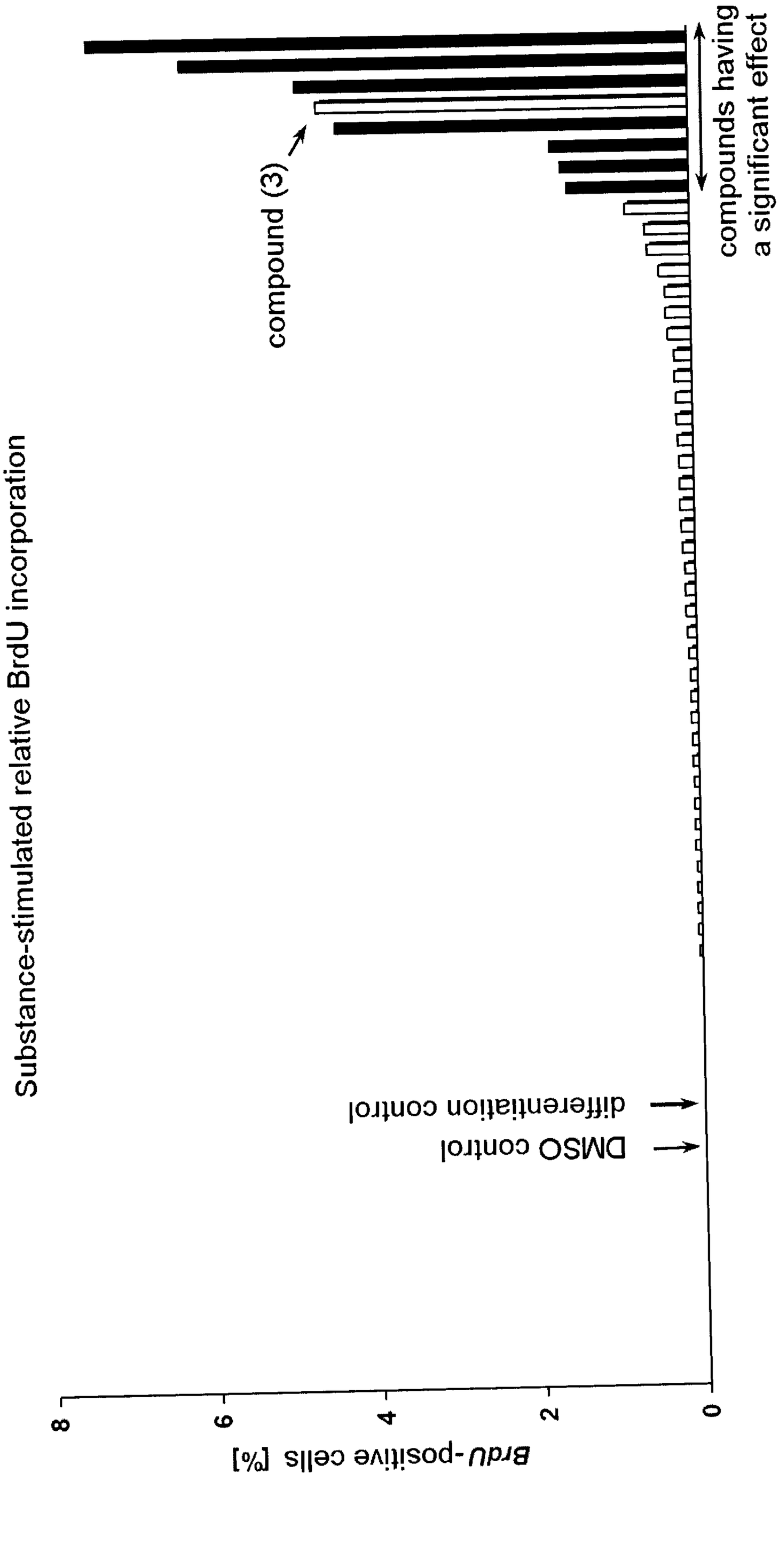
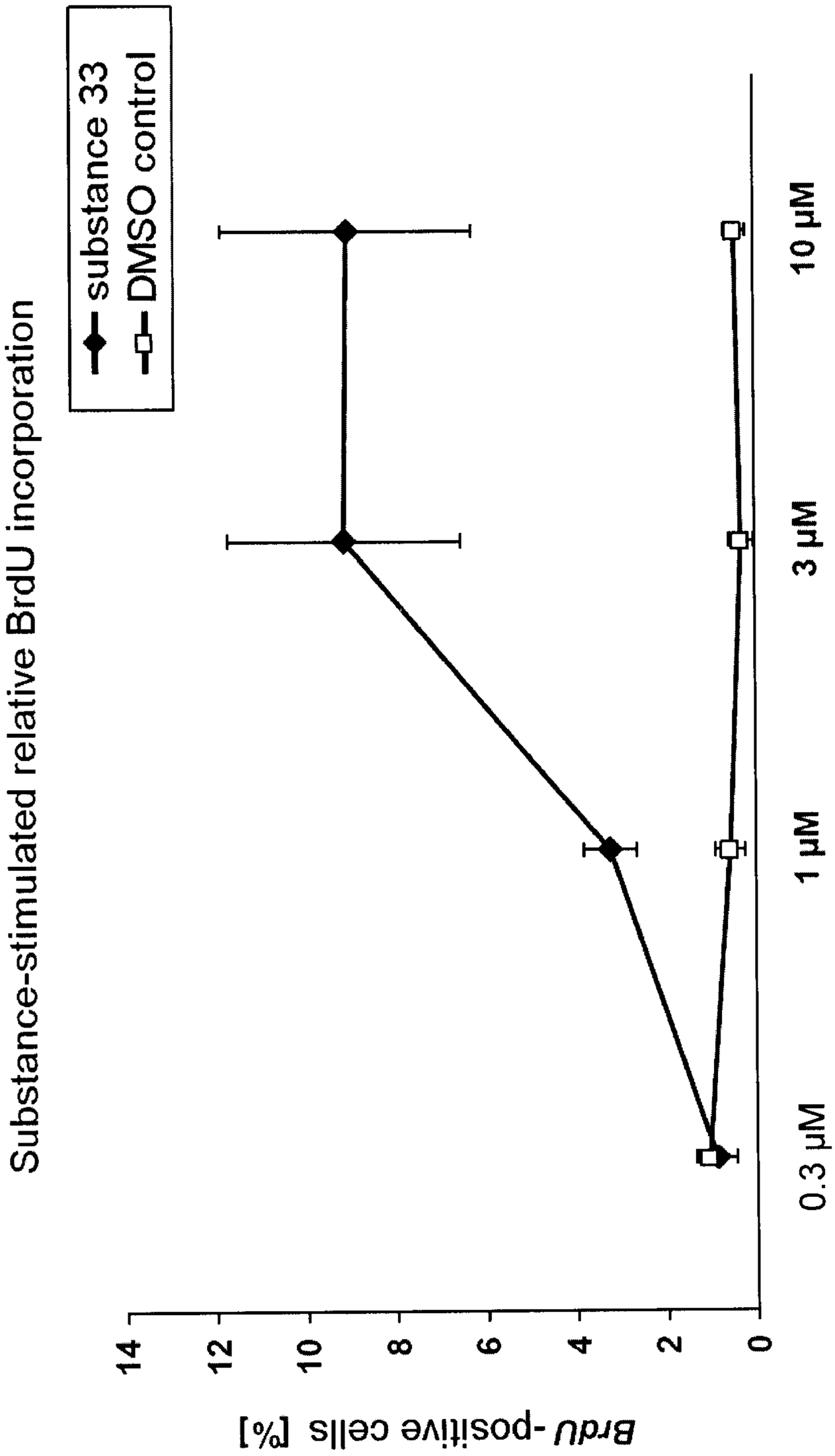

Substance-stimulated relative BrdU incorporation
compound (3)
compounds having a significant effect
DMSO control
differentiation control
BrdU-positive cells [%]
0
2
4
6
8

Figure 4


substance 33
DMSO control
Substance-stimulated relative BrdU incorporation
BrdU-positive cells [%]
0
2
4
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8
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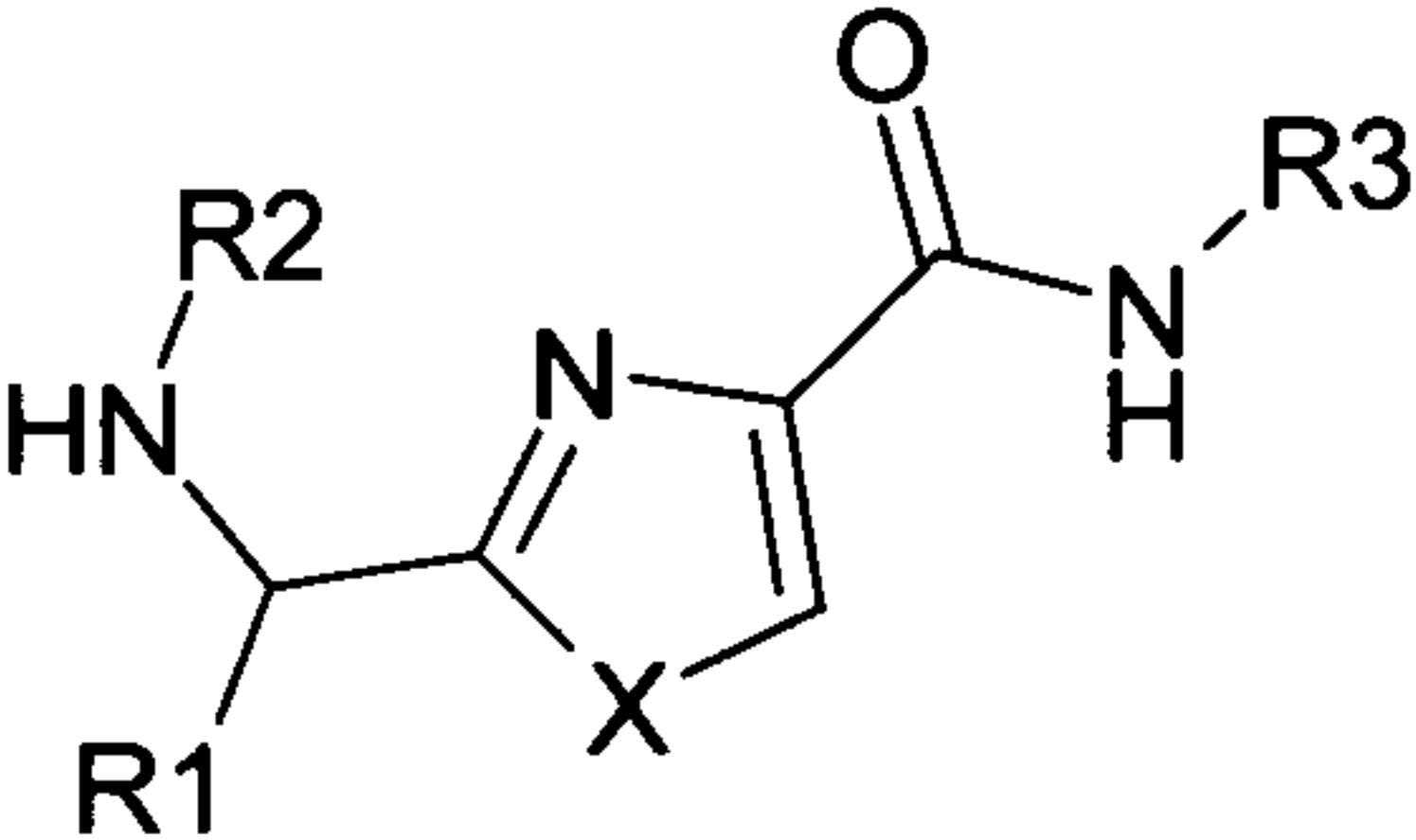
12
14
0.3 µM
1 µM
3 µM
10 µM
concentration

Figure 5



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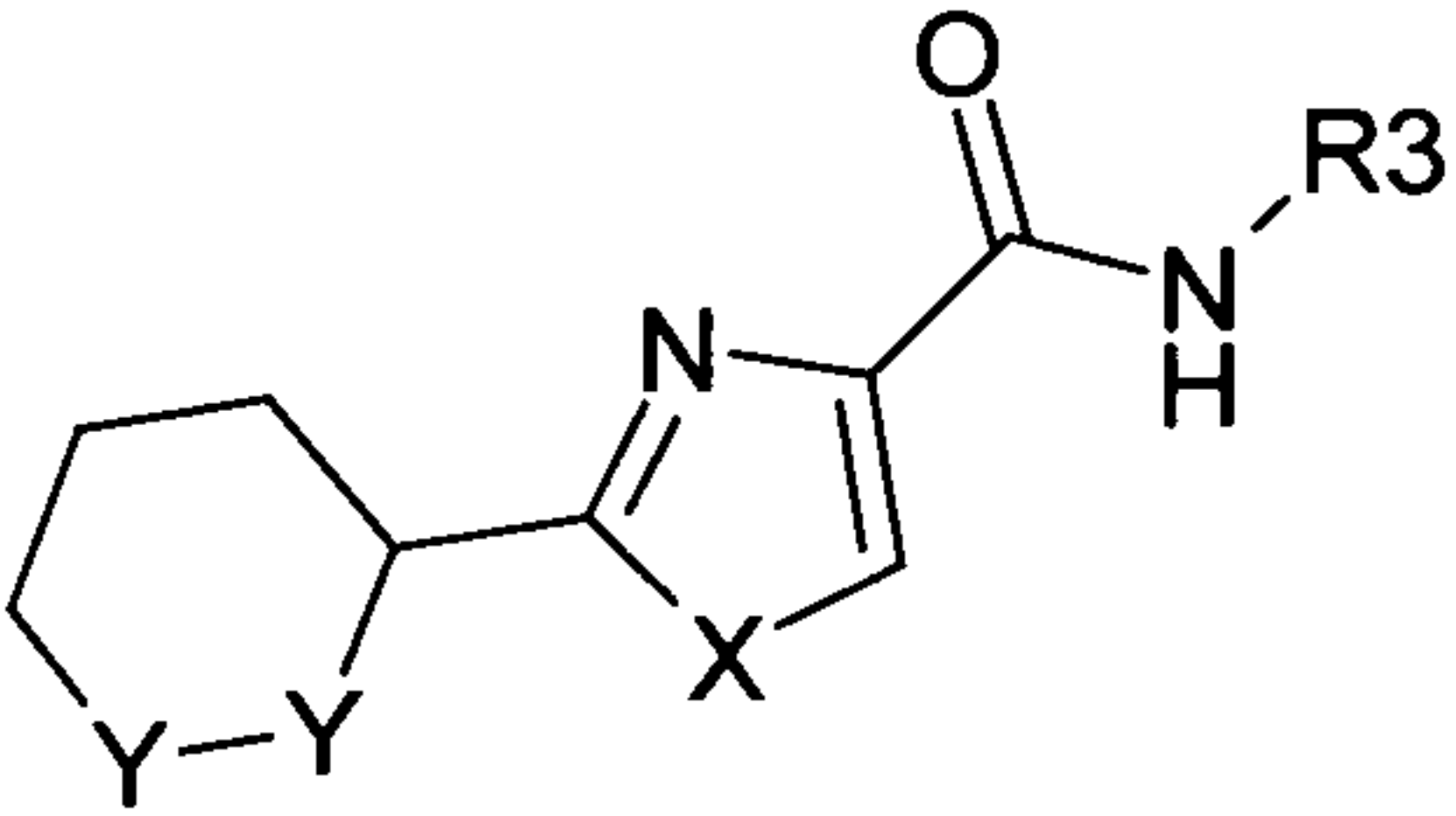

(2)

X = O, S