(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
13 November 2014 (13.11.2014)

WIPO | PCT

(10) **International Publication Number**
WO 2014/180455 Al

(51) **International Patent Classification:**
C07C 67/08 (2006.01) ***A61K 8/37*** (2006.01)
C07C 69/716 (2006.01) ***A61K 8/49*** (2006.01)
C07D 333/16 (2006.01) ***A61Q 19/00*** (2006.01)
C07D 295/088 (2006.01)

(21) **International Application Number:**
PCT/CZ20 14/000050

(22) **International Filing Date:**
5 May 2014 (05.05.2014)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
PV 2013-330 6 May 2013 (06.05.2013) CZ

(71) **Applicant: CONTIPRO BIOTECH S.R.O.** [CZ/CZ]; Dolni Dobrouc 401, 561 02 Dolni Dobrouc (CZ).

(72) **Inventors: KLEIN, Pavel;** Dolni Dobrouc 444, 56102 Dolni Dobrouc (CZ). **PRAVDA, Martin;** Podhomoli 1543, 56501 Chocen (CZ). **BUFFA, Radovan;** Kukorelliho 1495/2, 06601 Humenne (SK). **MATEJKOVA, Ilona;** Dukelska 307, 56201 Usti nad Orlici (CZ). **VELEBNY, Vladimir;** Sadova 1466, 56401 Zamberk (CZ).

(74) **Agent: DVORAKOVA, Martina;** Kania, Sedlak, Smola, Mendlovo namesti la, 603 00 Brno (CZ).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2014/180455 A1

(54) **Title:** METHOD OF PREPARATION OF 2-OXOGLUTARATE ESTER, PREPARATION CONTAINING 2-OXOGLUTARATE ESTER AND USE THEREOF

(57) **Abstract:** The invention relates to a method of preparation of 2-oxoglutarate ester, a preparation containing 2-oxoglutarate ester and use thereof for supporting collagen biosynthesis.

Method of preparation of 2-oxoglutarate ester, preparation containing 2-oxoglutarate ester and use thereof

Field of the Art

The invention relates to the method of preparation of 2-oxoglutarate ester, a preparation containing 2-oxoglutarate ester and a use thereof for supporting the biosynthesis of collagen.

Prior Art

Prolin is essential for the biosynthesis of collagen. Thereafter, post-translationally by means of prolyl-4-hydroxylase, prolin gives rise to hydroxyprolin which is necessary for the formation of collagen triple helix and for the mechanical properties thereof. Incomplete hydroxylation of prolin residues X-Pro-Gly in the repeating amino acid motives leads to errors in the synthesis of the collagen triple helix (Myllyharju, 2003). 2-oxoglutarate, the intermediate of the Krebs cycle, which is the substrate for prolyl-4-hydroxylase, is essential for the hydroxylation of prolin, together with Fe^{2+} ions and vitamin C. Therefore, the presence of said substances is crucial for collagen biosynthesis.

The use of native 2-oxoglutarate as the means of stimulation of collagen biosynthesis is known, wherein the positive effect of the substance was evidenced experimentally *in vitro* on the culture of fibroblasts and *in vivo on* hairless mice which were long UVB irradiated. External application of 1% 2-oxoglutarate together with propylen glycol and ethanol lead in the experimental animal to the reduction of consequences of actinic ageing of skin, including suppression of wrinkle formation (Son et al., 2007). Biological availability and effectivity of transdermal penetration of transdermal penetration of 2-oxoglutarate is herein achieved using propylen glycol-ethanol carrier which impairs the natural function of skin barrier by disrupting the surface structures thereof, which is disadvantageous. The Korean patent KR1219294 discloses a cosmetic composition effective in smoothing out wrinkles, containing non-modified 2-oxogiutarate and optionally a-lipoic acid.

The deficiency of said solution is the use of the non-modified molecule which, due to its hydrophilic character, has difficulties with getting over biological barriers and must be combined with substances (e.g. propylenglycol and ethanol), which facilitate the penetration of the non-modified molecule and thus enhance the biological availability thereof. However, these substances function in a way where they destroy the natural protective function of biological barriers, which is undesirable. Moreover, the cells react to the external addition of non-modified 2-oxoglutarate just by a slight and statistically insignificant increase of the production of collagen.

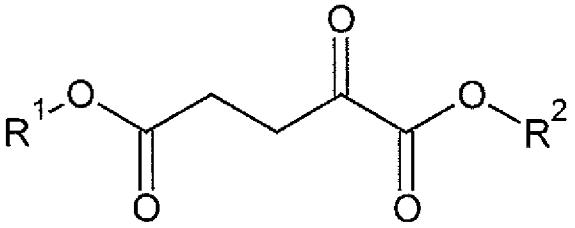
The esterified 2-oxoglutarate is a substance which is known and the effect thereof on pseudohypoxia control in cells has been disclosed (MacKenzie et al; 2007). The use of 2-oxoglutarate monoesters for the prevention of pseudohypoxia of cells is disclosed in US2009005437 (WO2006/016143). It is known that 2-oxocarboxylic acids are generally instable, therefore all up-to-date known processes of preparation of 2-oxoglutaric acid esters disclosed in the literature have been performed without any significant heating, in some cases even with cooling (WO2006/016143).

Summary of the invention

For hydrophobization of the molecule in order to make the molecule more biologically available for the cells, without the necessity of using further additives, this invention makes use of esterification of at least one carboxyl of 2-oxoglutarate by means of alcohol. The ester is, as the transport form, stable enough in physiological conditions for an easy penetration of the molecule through the physiological barriers. However, after entering the intracellular space it is quickly hydrolysed by the present enzymes to give the biologically active 2-oxoglutarate and biocompatible alcohols.

Moreover, compared to the effect of the simple 2-oxoglutarate, it was surprisingly found out that the esters of 2-oxoglutarate have a significant procollagen effect in cells exposed to oxidation stress due to e.g. age, inflammation, UV exposition, harmful chemical substances etc. Such effect is comparable to the effects of growth hormones.

The drawbacks of the prior art are overcome by the ester of 2-oxoglutarate according to the general formula I,



(I),

wherein

R^1 and R^2 are identical or different and are independently selected from the group comprising hydrogen, C_1-C_{30} alkyl, C_2-C_{30} alkenyl, C_4-C_{30} cycloalkyl, C_4-C_{30}

cycloalkenyl, C_1-C_{30} alkylaryl or C_1-C_{30} alkylheteroaryl, in which R^1 and R^2 optionally contain one or more identical or different heteroatoms selected from the group comprising N, O, S; and wherein R^1 and R^2 are not hydrogens at the same time; or pharmaceutically acceptable salts thereof;

for use in medicine, veterinary medicine, cosmetics, biotechnological applications or in tissue engineering for the stimulation of collagen biosynthesis, especially in cells exposed to oxidation stress.

2-oxoglutarate ester defined above is used for the production of a medical device, medicinal preparation, veterinary preparation, veterinary technical device, veterinary medicinal preparation or preparation serving as a component of media for cultivation and storage of cells, for tissue engineering or for biotechnological applications for the stimulation of collagen biosynthesis.

The preparations mentioned above may be used for stimulation of collagen biosynthesis *in vivo* in connective tissues, as well as. in biotechnological applications and in cell and tissue engineering.

The amount of the 2-oxoglutarate ester defined above in the preparation according to the invention is within the range of 0.01 %wt. to 10 %wt. with respect to the total weight of the preparation. Further, the preparation according to the invention contains physiologically acceptable additives selected from the group including acrylates/C10-C30 alkylacrylate crosslinked polymer, panthenol, glycerin, methylparaben, fatty acid esters of olive oil and sorbitan/cetearyl alcohol, sorbitan monolaurate, olive oil, glycerylmonostearate, squalene, butylparaben, macadam oil, hyaluronic acid, its derivatives and salts, alantoin, sodium polyacrylate, cetearylalcohol, coconut butter, isododecane, hydroxypropyl methylcellulose, sodium salt of carboxymethyl cellulose, xylitol, manitol, erythritol, vaseline, liquid paraffin, propylenglycol, stearomacrogol 100, stearomacrogol 1050, cetylalcohol, light liquid paraffin, propylparaben, cocoa oil, medium saturated triacyl glycerols, benzylacohol, sorbitans and polysorbates, butter pear butter, ceteareth-20, cetyl alcohol, shea butter, sorbitan stearate, span 20, 60, 80 stearic acid, stearyl palmitate, xanthan gum, 1,3-butanediol, almond oil, aloe vera, dimethicon, isopropylmyristate, polyethylenglycols (e.g. PEG-100 stearate, PEG 400), oil from grape stones or isohexadecane, which are commonly used for preparations especially in the field of cosmetics, medicine, veterinary medicine, tissue engineering and in biotechnological applications.

The preparation according to the invention is in the form of a solution, emulsion, suspension, gel, ointment, cream or dressing material.

The preparation according to the invention is preferably used **for stimulation** of collagen biosynthesis in cells exposed to the oxidation stress, containing collagen.

The oxidation stress is a non-equilibrium between the formation of the reactive oxygen (in other words also free radicals), which is formed as a by-product of oxygenation and metabolism, and the ability of the organism of fast degradation and detoxication of reactive intermediate products. It is involved in many diseases, such as atherosclerosis, Parkinson disease, heart failure or heart attack. Long lasting oxidation stress is, inter alia, the cause of skin ageing, the skin ceils being characterized by an increased error rate in post-translation modification of collagen. However, such error rate may also occur in any cells containing collagen e.g. by means of UV exposition, inflammation and other harmful substances, as mentioned above.

The preparation according to the invention is selected from the group including medical device, medicinal preparation, veterinary preparation, veterinary technical device, veterinary medicinal preparation or preparation serving as a component of media for cultivation and storage of cells, for tissue engineering and for biotechnological applications.

2-oxoglutarate ester defined above is preferably prepared by a method, the subject-matter of which being 2-oxoglutaric acid reacting with an alcohol according to the general formula R_3-OH, wherein R_3 is independently selected from the group including C_1-C_{30} alkyl, C_2-C_{30} alkenyl, C_4-C_{30} cycloalkyl, C_4-C_{30} cycloalkenyl, C_1-C_{30} alkylaryl or C_1-C_{30} alkylheteroaryl optionally containing one or more identical or different heteroatoms selected from the group including N, O, S; above the melting point of 2-oxoglutaric acid, preferably at temperatures 113.6 °C to 125 °C, more preferably at 120 °C.

According to a preferred embodiment of the method of the invention, the esterification reaction is carried out for 0.5 to 2 hours, preferably for 1 hour. The preferred amount of the alcohol in the reaction mixture is within the range of 2 to 3 molar equivalents with respect to the molar amount of 2-oxoglutaric acid, more preferably 2.4 molar equivalents with respect to the molar amount of 2-oxoglutaric acid.

The method of preparation of 2-oxoglutarate ester according to the invention is, as opposed to the processes known up to now, totally different. Since there is no need of neither solvents, catalysts, nor activating agents, the resulting mixture does not have to be isolated or purified, which facilitates the preparation itself very much and reduces the cost of the prepared material. The active substance which is used for the production of the preparation according to the invention is the crude reaction mixture.

The effectivity of the support of the collagen production was observed both in cells of juvenile donors, and in cells of senescent donors. The cells of senescent donors (age above 50 years) served as a model of cells exposed to the oxidation stress in which an increased error rate of post-translation modification of collagen is generally known.

Detailed Description of the Drawings

Fig. **1** represents the viability of the cells of 3T3 line in the presence of 5 various batches of hexyl-2-oxoglutarate (batch OG50 to OG54 prepared according to Example **1)** having the concentration of 50 - 500 µg / ml. For the measurement of cell viability, a test with neutral red was used.

Fig. 2 represents the cell viability of 3T3 line in the presence of 2-phenylethyl-2-oxoglutarate (batch OG47 prepared according to Example 2) having the concentration 50 - 500 µg ***I*** ml. The control was the solvent alone (dimethylsulfoxide; DMSO). For the measurement of cell viability, a test with neutral red was used.

Fig. 3 represents the influence of hexyl ester of 2-oxoglutarate prepared according to Example I on ***in vitro*** production of collagen by human dermal fibroblasts (NHDF) from child donors and donors older than 50years.

Fig. 4 represents the influence of 2-phenylethyl ester of 2-oxoglutarate prepared according to Example 2 on ***in vitro*** collagen production by human dermal fibroblasts (NHDF).

Fig. **5** represents the influence of 2-phenylethyl ester of 2-oxoglutarate prepared according to Example 2 on ***in vitro*** collagen production by human gingival fibroblasts (HGF).

Examples

The following examples of the embodiments of the invention should be understood as merely illustrative, without limiting the scope of the invention and the required protection in any way.

SE = esterification degree = nE /nK, defines how many ester groups fall on one molecule of the initial 2-oxoglutaric acid in average.

nE = molar amount of all ester hydrogens (alkyl-CH_2-0-CO-), expressed by integral of the signals in ^1H NMR spectrum within the interval 4.0 to 4.5 ppm

nK = molar amount of all aipha-keto hydrogens (-O-CO-CO **-CH2-)** expressed by integral of the signals in ^{1}H NMR spectrum within the interval 3.0 to 3.3 ppm

^{1}H NMR spectra measured in deuterated chloroform with a device having the frequency of 500 MHz, chemical shifts were calibrated with respect to the inner standard of deuterated 3-trimefhyl-silyl propane acid.

Example 1

Preparation of hexylesters of 2-oxoglutarate.

2-Oxogjutaric acid (1 g) was melted at the temperature of 115 °C. Then 2 equivalents of 1-hexanol were slowly added and the mixture was stirred for 2 hours at the temperature of 115 °C. The resulting product (20 mg) was then dissolved in deuterated trichloromethane (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.4.

Example 2

Preparation of 2-phenethylesters of 2-oxoglutarate.

2-Oxoglutaric acid (lg) was melted at the temperature of 115 °C. Then 2 equivalents of 2-phenylethyl alcohol were slowly added and the mixture was stirred for 0.5 hour at the temperature of 125 °C. The resulting product (20 mg) was then dissolved in deuterated trichloromethane (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy. SE = 1.9 .

Example 3

Preparation of octylesters of 2-oxoglutarate.

2-Oxoglutaric acid (1g) was melted at the temperature of 115 °C. Then 2 equivalents of 1-octanol were slowly added and the mixture was stirred for 1 hour at the temperature of 125 °C. The resulting product (20 mg) was then dissolved in deuterated trichloromethane (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.5.

Example 4

Preparation of 4-(thiophene-2-yl) butyl esters of 2-oxoglutarate.

2-Oxoglutaric acid (lg) was melted at the temperature of 115 °C. Then 3 equivalents of 4-(thiophene-2-yl)butan-l-ol were slowly added and the mixture was stirred for 1 hour at the temperature of 120 °C. The resulting product (20 mg) was then dissolved in deuterated chloroform (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.4 .

Example 5

Preparation of 4-(l-methylpiperidiniitm-l-yl) butyl esters of 2-oxogiutarate.

2-Oxoglutaric acid (1g) was melted at the temperature of 115 °C. Then 2 equivalents of4-(l-methylpiperidinium-l-y!)butan-l-ol were slowly added and the mixture was stirred for 1 hour at the temperature of 120 °C. The resulting product (20 mg) was then dissolved in deuterated chloroform (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.3.

Example 6

Preparation of oct-7-ene-l-yl esters of 2-oxogiutarate.

2-Oxoglutaric acid (lg) was melted at the temperature of 115 °C. Then 2.6 equivalents of oct-7-ene-l-ol were slowly added and the mixture was stirred for 1 hour at the temperature of 120 °C. The resulting product (20 mg) was then dissolved in deuterated chloroform (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.6.

Example 7

Preparation of 4-(cyclohex-l-ene-l-yl)butyl esters of 2-oxoglutarate.

2-Oxoglutaric acid (lg) was melted at the temperature of 115 °C. Then 2.8 equivalents of 4-(cyclohex-l-ene-l-yI)butan-l-ol were slowly added and the mixture was stirred for 1 hour at the temperature of 120 °C. The resulting product (20 mg) was then dissolved in deuterated chloroform (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.7.

Example 8

Preparation of 4-cyclohexylbutyl esters of 2-oxoglutarate.

2-Oxoglutaric acid (lg) was melted at the temperature of 115 °C. Then 2.4 equivalents of 4-cyclohexylbutan-l-ol were slowly added and the mixture was stirred for 1 hour at the temperature of 120 °C. The resulting product (20 mg) was then dissolved in deuterated chloroform (0.8 ml) and analysed by means of ^{1}H NMR spectroscopy, SE = 1.5.

Example 9

The cosmetic preparation was prepared by mixing the individual components identified below together.

The composition of the cosmetic preparation in the form of a cream

	(wt./wt.)
1) triglycerides of octanoic acid and decanoic acid (Triglyceride, MakingCosmetics, USA)	12 %
2) glyceryl monostearate, PEG 100 stearate (CreamMaker Blend, Making Cosmetics, USA)	5 %
3) sodium aerylate, copolymer of acryloyl dimethyl taurate, isohexadecane, polysorbate 80 (GelMaker EMU, Making Cosmetics, USA)	2 %
4) tocopheryl acetate	1 %
5) glycerin	4 %
6) hexylester of 2-oxoglutaric acid	5 %
7) benzylalcohol, DHA	0.8 %
8) perfume	0.2 %
9) distilled water	75 % .

Example 10

The cosmetic preparation was prepared by mixing the individual components identified below together.

The composition of the cosmetic preparation in the form of a gel

	wt./wt.
1) sodium hyaluronan	0.7 %
2) distilled water	88.25 %
3) PEG-40 hydrogenated castor oil	0.6 %
4) ethylalcohol 96%	2 %
5) perfume	0.05 %
6) 1,3-butandiol	2 %
7) sodium lactate, sodium salt of pyrolidoncarbonic acid, glycine, fructose, urea, niacinamide, inositol, sodium benzoate, lactic acid (Lactil, Evonik)	3 %
9) disodium salt of EDTA	0.05 %
10) D-panthenol	0.3 %

11) glycerin, copolymer of glyceryl acrylate/acrylic acid, propylene glycol (Lubrajel MS, Ashland)	2 %
hexylester of 2-oxoglutaric acid in PEG400 (1%)	1%
methylchloro isothiazolinone, methyl isothiazolinone (Isocii PC, Lonza AG)	0.05%

Example 11

The cosmetic preparation was prepared by mixing the individual components identified below together.

The composition of the cosmetic preparation in the form of a cream

	wt./wt.
1) Steareth-2 (PEG-2 stearylether)	2 %
2) Steareth-21 (PEG-21 stearylether)	2 %
3) PPG-15 stearylether	9 %
4) palmitic acid, stearic acid (Cutina FS 45, Cognis)	1.5 %
5) dimethicone (silicone oil)	0.5 %
6) isopropyl myristate	3 %
7) triglycerides of octanoic acid and decanoic acid (Triglyceride, MakingCosmetics, USA)	2 %
8) squalene	3 %
9) cetylalcohol	2 %
10) glycerylstearate	1 %
11) hexylester of 2-oxoglutaric acid	10 %
12) glycerin	4 %
13) D-panthenol	0.5 %
14) distilled water	59.25 %
15) methylchloro isothiazolinone, methyl isothiazolinone (isocii PC, Lonza AG)	0.05 %
16) perliime	0.2 %

Example 12

Cell viability test

Fig. 1 shows the cell viability (fibroblasts of 3T3 line) in the presence of hexylester of 2-oxoglutaraie (50 - 500 µg / ml) prepared according to Example 1. Fig. 2 shows cell viability (fibroblasts of 3T3 line) in the presence of 2-phenylethyl ester of 2-oxoglutarate (50 - 500 µg/ml) prepared according to Example 2. The viability was measured by the standard neutral red method. The results document that the tested esters are not cytotoxic.

Example 13

Comparative *in vitro* test of the effects of hexylester of 2-oxoglutarate (Fig. 3) and 2-phenylethyl (Fig. 4 and 5) on the production of total collagen

Fig. 3 shows the test results of the effect of 2-oxoglutarate hexylester (1000 µg / ml) prepared according to Example 1 on *in vitro* production of the total collagen by human dermal fibroblasts (NHDF) obtained from human donors (age 50 - 65 years - senescent cells) and also (age 8-11 years - juvenile cells). Fig. 4 shows the test results of the effect of 2-phenylethyl ester of 2-oxoglutarate (10 - 1000 µg / ml) prepared according to Example 2 on *in vitro* production of the total collagen by human dermal fibroblasts (NHDF) obtained from human donors (age 50 - 65 years). Fig. 5 shows the test results of the effect of 2-phenylethyl ester of 2-oxoglutarate (10 - 1000 µg / ml) prepared according to Example 2 on *in vitro* production of the total collagen by human gingival fibroblasts (HGF) obtained from human donors (females, 50 - 60 years).

In order to monitor the collagen production, the cells were seeded in 96-wells culture panels in seeding density of 10 000 cells/well and they were cultivated for at least 12 hours in the medium (DMEM) with 10% of fetal bovine serum. Thereafter, the medium was changed for DMEM containing proline (400 mg/1) and vitamin C (trisodium 2-phospho-L-ascorbate; 480 mg/1). After further 20-24 hours, the medium was changed for DMEM with the tested substances (exposition of 24 hours). The total production of collagen was tested in the medium by the method of specific coloration of collagen by the saturn red solution F3B200 in picric acid (Walsh et al., 1992; Heng et al. 2006).

All experiments on cell cultures were repeated at least 3x, while keeping the above described experimental conditions. The obtained data were evaluated statictically by variance analysis, Scheffe's test was used for comparing the individual groups (multiple range test). The results illustrate that 2-oxoglutarate hexylester prepared according to Example 1 and 2-

oxoglutarate 2-phenylethyl ester prepared according to Example 2 increased significantly the total collagen production by human dermal fibroblasts. 2-phenylethyl ester of 2-oxoglutarate prepared according to Example 2 increased significantly the total collagen production by human gingival fibroblasts. The surprising effect is apparent not only when compared to the control (non-effected cells), but also when compared to the non-modified 2-oxoglutarate.

According to the published experimental data (Son et al. 2007), in case of senescent cells no statistically significant pro-collagen effect of the non-modified 2-oxoglutarate was proven. Moreover, as seen in Fig. 3, the effect of 2-oxoglutarate and 2-oxoglutarate ester on collagen production in cells of juvenile donors is statistically comparable.

The used literature

Heng EC, Huang Y, Black SA Jr, Trackman PC: CCN2, connective tissue growth factor, stimulates collagen deposition by gingival fibroblasts via module 3 and alpha6- and betal integrins. *J Cell Biochem.* 2006;98(2):409-20.

Vandenheuvel FA: Structure of membranes and role of lipids therein. *Adv Lipid Res.* 1971;9:161-248.

Myllyharju J: Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. *Matrix Biol.* 2003;22(1): 15-24

Son ED, Choi GH, Kim H, Lee B, Chang IS, Hwang JS: Alpha-ketoglutarate stimulates procollagen production in cultured human dermal fibroblasts, and decreases UVB-induced wrinkle formation following topical application on the dorsal skin of hairless mice. *Biol Pharm Bull.* 2007;30(8):1 395-9.

MacKenzie ED, Selak MA, Tennant DA, Payne LJ, Crosby S, Frederiksen CM, Watson DG, Gottlieb E: Cell-permeating alpha-ketoglutarate derivatives alleviate pseudohypoxia in succinate dehydrogenase-deficient cells. *Mol Cell Biol.* 2007;27(9):3282-9.

Benson, H. A. E., Transdermal Drug Delivery: Penetration Enhancement Techniques. In *Current Drug Delivery,* Bentham Science Publishers Ltd.: 2005; Vol. 2, pp 23-33.

Rosenblat G, Perelman N, Katzir E, Gal-Or S, Jonas A, Nimni ME, Sorgente N, Neeman I: Acylated ascorbate stimulates collagen synthesis in cultured human foreskin fibroblasts at lower doses than does ascorbic acid. *Connect Tissue Res.* 1998;37(3-4):303-l 1.

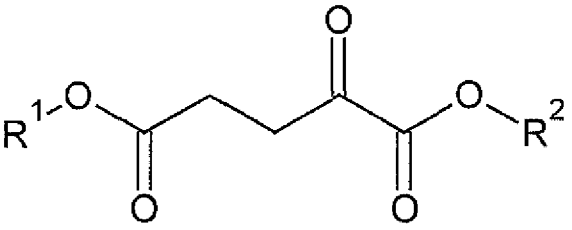
Walsh BJ, Thornton SC, Penny R, Breit SN: Microplate reader-based quantitation of collagens. *Anal Biochem.* 1992:203(2): 187-90.

Gottlieb E, Selak MA, Mackenzie ED, Watson DG: ALPHA-KETOGLUTARATES AND THEIR USE AS THERAPEUTIC AGENTS. WO/2006/016143, 2006.

Son ED, Lee SH, Kim HK, Park CM, Hwang JS, Chang TS: ANTI-WRINKLE COSMETIC COMPOSITION CONTAINING ALPHA-KETOGLUTARATE. KR 2008001 1543 (A), 2008

CLAIMS

1. A method of preparation of 2-oxoglutarate ester according to the general formula I



(I),

wherein

R^1 and **R^2** are identical or different and are independently selected from the group comprising hydrogen, **C1-C30** alkyl, **C2-C30** alkenyl, C_4-C_{30} cycloalkyl, **C4-C30** cycloalkenyl, **C_1-C30** alkylaryl or **C_1-C30** alkylheteroaryl in which **R^1** and **R^2** optionally contain one or more identical or different heteroatoms selected from the group comprising N, O, S; and wherein **R^1** and **R^2** are not hydrogens at the same time; or pharmaceutically acceptable salts thereof,

characterised by that 2-oxoglutaric acid reacts with alcohol according to the general formula **R^3OH,** wherein **R^3** is independently selected from the group comprising **C1-C30** alkyl, **C_2-C30** alkenyl, **C_4-C_{30}** cycloalkyl, **C_4-C_{30}** cycloalkenyl, **C1-C30** alkylaryl or **C1-C30** alkylheteroaryl optionally containing one or more identical or different heteroatom selected from the group comprising N, **O,** S; above the melting point of 2-oxoglutaric acid.

2. The method of preparation of 2-oxoglutarate ester according to the general formula Γ according to claim 1, **characterised by that R^J** is selected from the group comprising hexyl, octyl, phenylethyi.

3. The method according to any of claims 1 and 2, **characterised by that** 2-oxoglutaric acid reacts with the alcohol at the temperature of 1 13.6 °C to 125 °C.

4. The method according to claim 3, **characterised by that** the temperature is 120 °C.

5. The method according to any of claims 1 to 4, **characterised by that** 2-oxoglutaric acid reacts with the alcohol for 0.5 to 2 hours.

6. The method according to claim 5, **characterised by that** 2-oxoglutaric acid reacts with the alcohol for 1 hour.

7. The method according to any of claims 1 to 6, **characterised by that** the amount of alcohol in the reaction mixture is within the range of 2 to 3 molar equivalents with respect to the molar amount of 2-oxoglutaric acid.

8. The method according to claim 7, **characterised by that** the amount of alcohol in the reaction mixture is 2.4 molar equivalents with respect to the molar amount of 2-oxoglutaric acid.

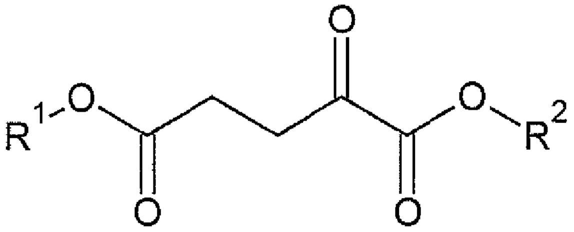
9. 2-oxoglutarate ester prepared by the method of any claim 1 to 8 for use in cosmetics, biotechnology applications or in tissue engineering for stimulation of collagen biosynthesis.

10. 2-oxoglutarate ester according to claim 9 for use for stimulation of collagen biosynthesis in cells exposed to oxidation stress.

11. Use of 2-oxoglutarate ester prepared by the method of any claim 1 to 8 for the production of a medical device, medicinal preparation, veterinary preparation, veterinary technical device, veterinary medicinal preparation or preparation serving as a component of media for cultivation and storage of cells, for tissue engineering or for biotechnological applications for stimulation of collagen biosynthesis.

12. The use of 2-oxoglutarate ester according to claim 11 for stimulation of collagen biosynthesis in cells exposed to oxidation stress.

13. 2-oxoglutarate ester according to the general formula **I**



(I),

wherein

R^1 and R^2 are identical or different and are independently selected from the group comprising hydrogen, C_1-C_{30} alkyl, C_2-C_{30} alkenyl, C_4-C_{30} cycloalkyl, C_4-C_{30} cycloalkenyl, C_1-C_{30} alkylaryl or C_1-C_{30} alkylheteroaryl in which R^1 and R^2 optionally contain one or more identical or different heteroatoms selected from the group comprising N, O, S; and wherein R^1 and R^2 are not hydrogens at the same time; or pharmaceutically acceptable salts thereof, for use in cosmetics, biotechnological applications or tissue engineering for stimulation of collagen synthesis.

14. 2-oxoglutarate ester according to claim 13 for use in stimulation of collagen biosynthesis in cells esposed to oxidation stress.

15. A preparation for stimulation of collagen biosynthesis **characterized in that** it contains 2-oxoglutarate ester according to claim 13.

16. The preparation according to claim 15 **characterized in that** the amount of 2-oxoglutarate ester is within the range of 0.01 %wt. to 10 %wt. with respect to the weight of the preparation.

17. The preparation according to any of claims 15 or 16, **characterized in that** it further contains physiologically acceptable substances.

18. The preparation according to claim 17, **characterized in that** the physiologically acceptable substance is selected from the group comprising acrylates/C_{10}-C_{30} alkylacrylate crosslinked polymer, panthenol, glycerin, methylparaben, esters of olive oil fatty acids and sorbitan/cetearyl alcohol, sorbitan monolaurate, olive oil, glyceryl monostearate, squalene, butylparaben, macadam oil, hyaluronic acid, derivatives and salts thereof, alantoin, sodium polyacrylate, cetearyl alcohol, coconut butter, isododecan, hydroxypropyl methylcellulose, sodium salt of carboxymethyl cellulose, xylitol, manitol,

erythriioi, vaseline, liquid paraffin, propylenglycoi, stearomacrogol 100, stearomacrogo[1050, cetylalcohol, light liquid paraffin, propylparaben, cocoa oil, medium saturated triacylglycerols, benzylacohol, sorbitans and polysorbates, alligator pear butter, ceteareth-20, cetyl alcohol, shea butter, sorbitan stearate, span 20, 60, 80 stearic acid, stearyl palmitate, xanthan gum, 1,3-butanediol, almond oil, aloe vera, dimethicon, izopropylmyristate, polyethylengiycols, grape seed oil or isohexadecane.

19. The preparation according to any of claims 15 to 18, **characterized** in **that** it is in the form of a solution, emulsion, suspension, gel, ointment, cream or dressing material.

20. The preparation according to any of claims 15 to 19 for use in stimulation of collagen biosynthesis in cells exposed to oxidation stress.

21. The preparation according to any of claims 15 to 20, **characterized** in **that** it is selected from the group comprising a medical device, medicinal preparation, veterinary preparation, veterinary technical device, veterinary medicinal preparation or preparation serving as a component of media for cultivation and storage of cells, for tissue engineering and for biotechnological applications.

22. A use of 2-oxoglutarate ester according to claim 13 or 14 for the production of a medical device, medicinal preparation, veterinary preparation, veterinary technical device, veterinary medicinal preparation or preparation serving as a component of media for cultivation and storage of cells, for tissue engineering or lor biotechnological applications for the stimulation of collagen biosynthesis.

23. The use of 2-oxoglutarate ester according to claim 22 for stimulation of collagen biosynthesis in cells exposed to oxidation stress.

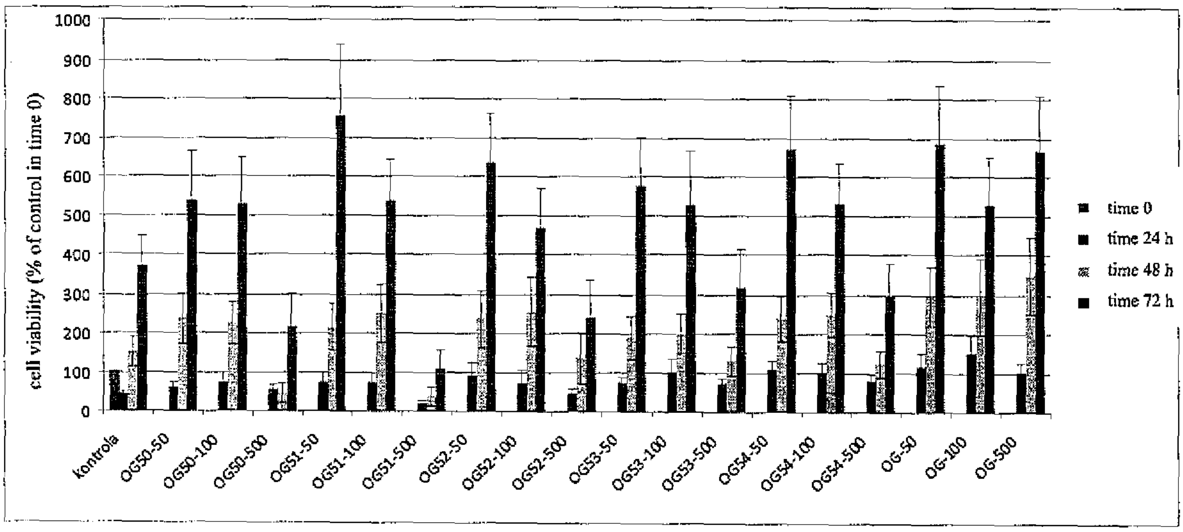

cell viability (% of control in time 0)
1000
900
800
700
600
500
400
300
200
100
0
kontrola
OG50-50
OG50-100
OG50-500
OG51-50
OG51-100
OG51-500
OG52-50
OG52-100
OG52-500
OG53-50
OG53-100
OG53-500
OG54-50
OG54-100
OG54-500
OG-50
OG-100
OG-500
time 0
time 24 h
time 48 h
time 72 h

Fig. 1

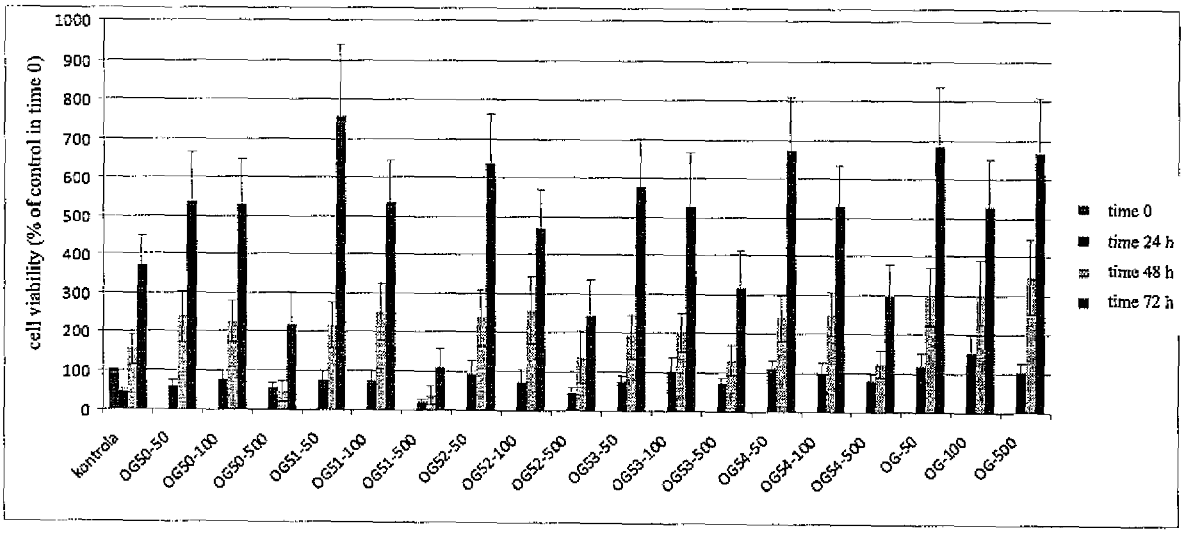
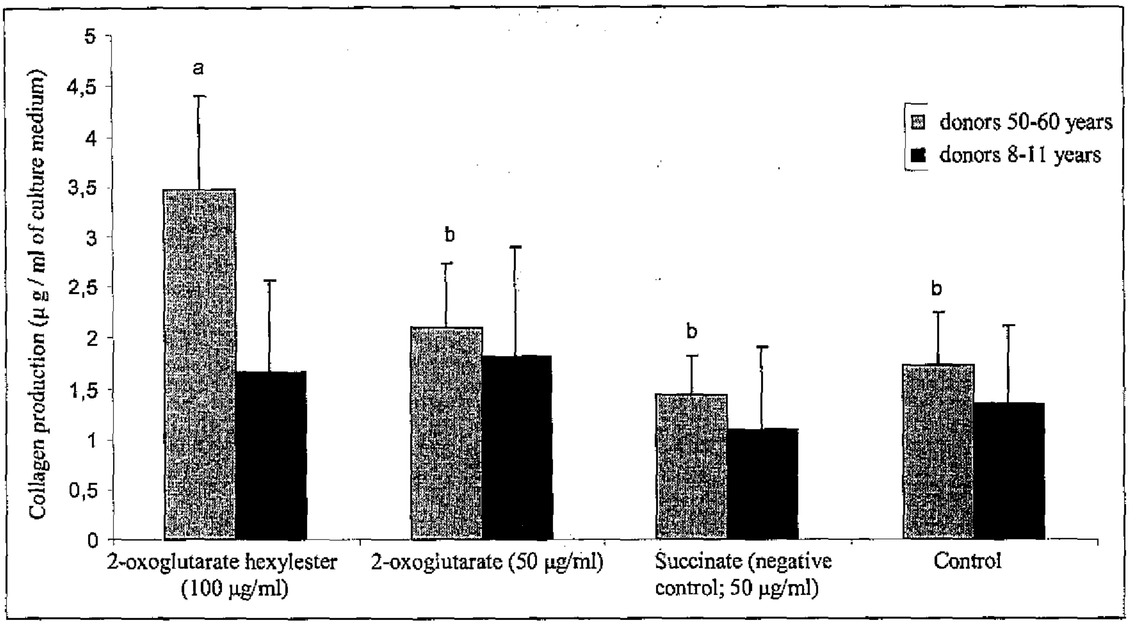
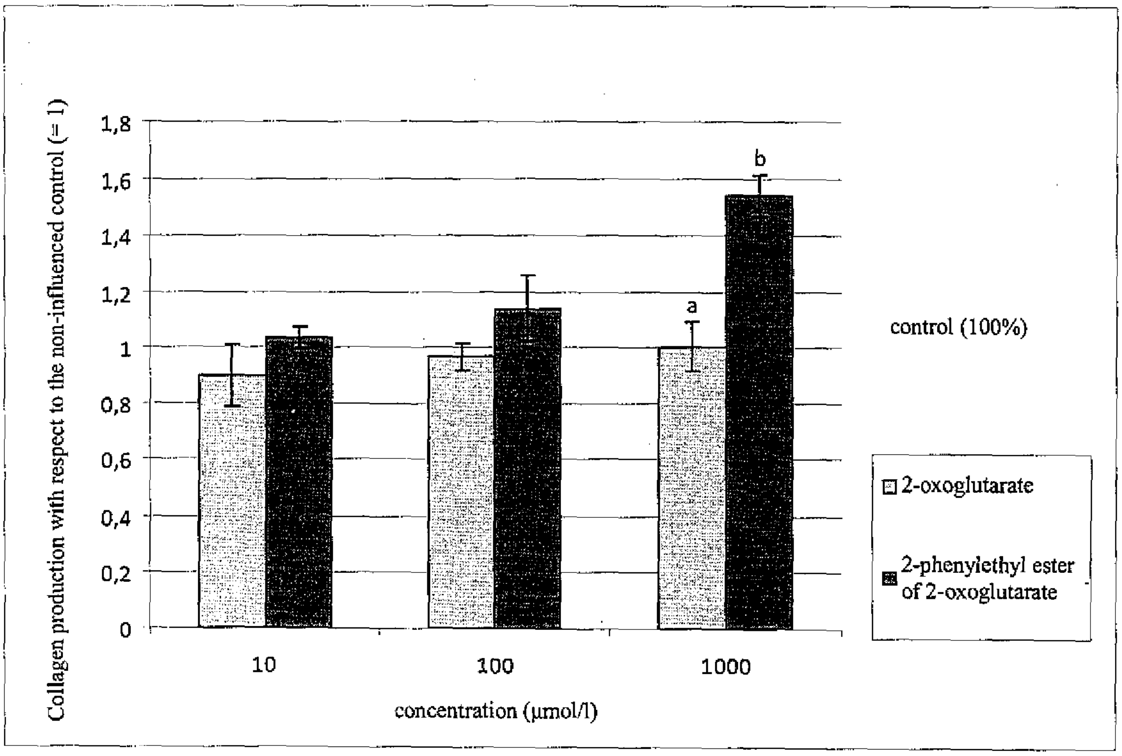

cell viability (% of control in time 0)
1000
900
800
700
600
500
400
300
200
100
0
kontrola
OG50-50
OG50-100
OG50-500
OG51-50
OG51-100
OG51-500
OG52-50
OG52-100
OG52-500
OG53-50
OG53-100
OG53-500
OG54-50
OG54-100
OG54-500
OG-50
OG-100
OG-500
time 0
time 24 h
time 48 h
time 72 h

Fig. 2



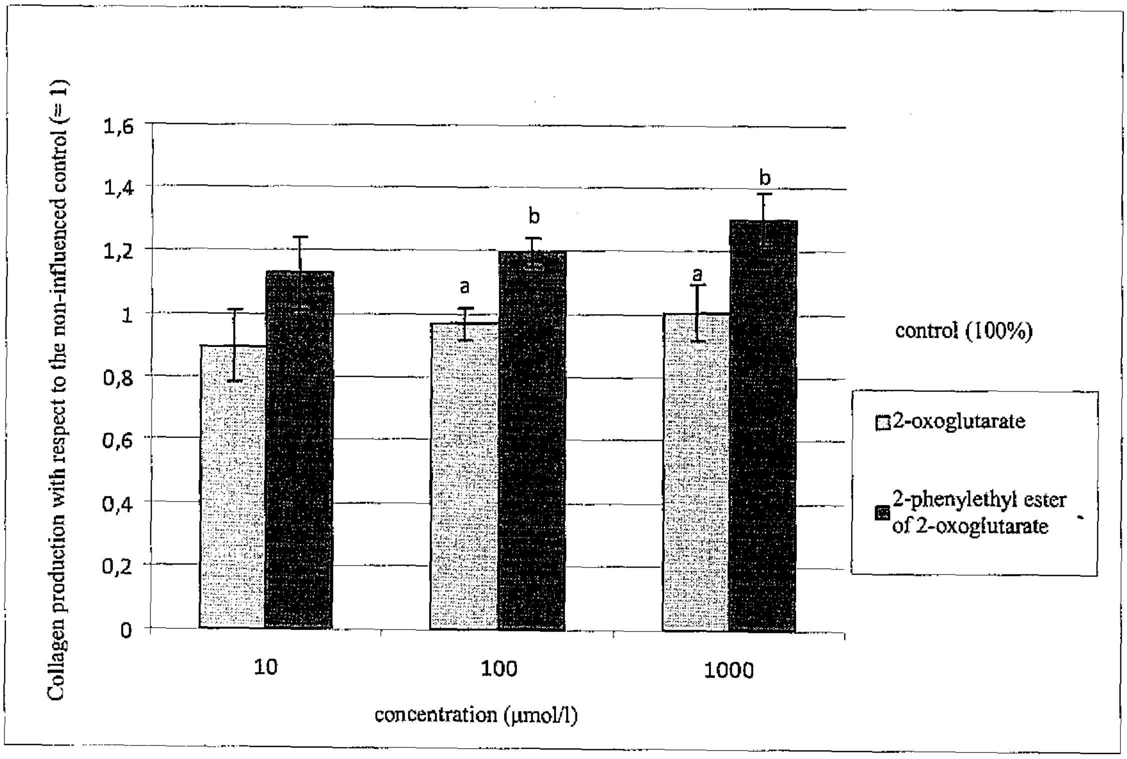
a,b : p<0,01; Succinate used as a negative control

Fig. 3


Collagen production with respect to the non-influenced control (= 1)
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
b
a
control (100%)
10
100
1000
concentration (µmol/l)
2-oxoglutarate
2-phenylethyl ester of 2-oxoglutarate

a,b : $p<0,01$

Fig. 4


Collagen production with respect to the non-influenced control (= 1)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
10
100
1000
concentration (µmol/l)
a
b
a
b
control (100%)
☐2-oxoglutarate
■2-phenylethyl ester of 2-oxoglutarate

a,b : p<0,01

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2014/000050

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07C67/08 C07C69/716 C07D333/16 C07D295/088 A61K8/37
A61K8/49 A61Q19/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07C C07D A61K A61Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 303 523 A1 (NABISCO BRANDS INC [US]) 15 February 1989 (1989-02-15) example 9 -----	1-10, 13, 14
X	EP 1 541 174 A1 (ISHIBASHI MICHIO [JP]) 15 June 2005 (2005-06-15) claims 3,5,10,12,23,25,32,35 paragraphs [0011], [0023], [0061], [0088], [0095] paragraph [0098] - paragraph [0102] -----	9-23
X	WO 2006/016143 A1 (CANCER REC TECH LTD [GB]; GOTTLIEB EYAL [GB]; SELAK MARY A [GB]; MACKE) 16 February 2006 (2006-02-16) cited in the application claim 33 page 60 - page 63 -----	9, 10, 13, 14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 8 August 2014	Date of mailing of the international search report 18/08/2014
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer van Bergen, Marc

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/CZ2014/000050

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0303523 Al	15-02-1989	AU 2422488 A	09-03-1989
		BR 8807164 A	17-10-1989
		CA 1322200 C	14-09-1993
		CN 1031367 A	01-03-1989
		EP 0303523 Al	15-02-1989
		FI 891732 A	12-04-1989
		JP H02500522 A	22-02-1990
		NZ 225825 A	29-01-1991
		PT 88270 A	30-06-1989
		US 4830787 A	16-05-1989
		W0 8901293 Al	23-02-1989
		ZA 8806033 A	26-04-1989
EP 1541174 Al	15-06-2005	AU 2003264406 Al	30-04-2004
		EP 1541174 Al	15-06-2005
		EP 1950563 A2	30-07-2008
		US 2006014709 Al	19-01-2006
		W0 2004024185 Al	25-03-2004
W0 2006016143 Al	16-02-2006	AU 2005271053 Al	16-02-2006
		CA 2574597 Al	16-02-2006
		EP 1778616 Al	02-05-2007
		JP 2008509209 A	27-03-2008
		US 2009005437 Al	01-01-2009
		W0 2006016143 Al	16-02-2006