Technical University of Denmark

DTU

Hyperpolarized 1-13c-1,1-bis(acetoxy(methyl))-2,2'-cyclopropane as metabolic marker for mr

Lerche, Mathilde Hauge; Jensen, Pernille Rose; Karlsson, Magnus; Napolitano, Roberta; Cabella, Claudia; Miragoli, Luigi; Colombo Serra, Sonia; Tedoldi, Fabio

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

DTU Library
Technical Information Center of Denmark

(54) Title: HYPERPOLARIZED 1-13C-1,1-BIS(ACETOXY(METHYL))-2,2'-CYCLOPROPANE AS METABOLIC MARKER FOR MR

(57) Abstract: 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane of formula (I): The compound can be hyperpolarized and used as a contrast agent in ^{13}C Magnetic Resonance diagnostic technique (^{13}C-MR) for the diagnosis of tumor.

WO 2015/000838 A1

Hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane as metabolic marker for MR

FIELD OF THE INVENTION

The invention relates to the field of Magnetic Resonance (MR), in particular to novel diagnostic media comprising hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane and to a
5   diagnostic method exploiting said molecule as MR tracer.

BACKGROUND OF THE INVENTION

Magnetic resonance imaging (MRI) is a technique that has become particularly attractive to physicians as images of a patient's body or parts thereof can be obtained in a non-invasive way and without exposing the patient and the medical personnel to a potentially harmful radiation such as X-
10   rays. Because of its high quality images and good spatial and temporal resolution, MRI is a favourable imaging technique for imaging soft tissue and organs. MRI may be carried out with or without MR contrast agents. However, contrast-enhanced MRI usually enables the detection of much smaller tissue changes, which makes it a powerful tool for the detection of early stage tissue changes like for instance small tumors or metastases.

15   MRI using hyperpolarized molecules is an emerging technique. WO 9935508 discloses a method of MR investigation of a patient using a hyperpolarized solution of a high T_1 agent as MRI contrast agent. The term "hyperpolarization" means enhancing the nuclear polarization of the NMR active nuclei present in the agent, i.e. nuclei with non-zero nuclear spin, preferably ^{13}C- or ^{15}N-nuclei, and thereby amplifying the MR signal intensity by a factor of hundred and more. When using a
20   hyperpolarized ^{13}C- and/or ^{15}N-enriched high T_1 agent, there will be essentially no interference from background signals as the natural abundance of ^{13}C and/or ^{15}N is negligible and thus the image contrast will be advantageously high. The main difference between conventional MRI contrast agents and these hyperpolarized high T_1 agents is that in the former changes in contrast are caused by affecting the relaxation times of water protons in the body whereas the latter class of agents can be
25   regarded as non-radioactive tracers, as the signal obtained arises solely from the agent. When hyperpolarization is obtained via a microwave assisted transfer between unpaired electrons and the nuclei used as MR probes, the techniques is referred as Dynamic Nuclear Polarization (DNP).

A variety of possible high T_1 agents for use as MR imaging agents are disclosed in WO9935508, including non-endogenous and endogenous compounds. As examples of the latter, intermediates in
30   normal metabolic cycles are mentioned which are said to be preferred for imaging metabolic activity. By *in vivo* imaging of metabolic activity, information of the metabolic status of a tissue may be obtained and said information may for instance be used to discriminate between healthy and diseased tissue.

For example, WO 2009077575 discloses a method of ^{13}C-MR detection using an imaging
35   medium comprising hyperpolarized ^{13}C-fumarate, in order to investigate both the citric acid and the urea cycles by detecting ^{13}C-malate and optionally ^{13}C-fumarate and/or ^{13}C-succinate signals. The metabolic profile generated in a preferred embodiment of the method provides information about the

metabolic activity of the body and part of the body under examination and said information may be used in a subsequent step for, e.g. identifying diseases. Such a disease is preferably cancer since tumor tissue is usually characterized by an altered metabolic activity. As a technical aspect, if the compounds to be polarized crystallize upon freezing or cooling their solution, a glass-forming additive

5   must be added to the solution.

Dynamic nuclear polarization (DNP) has been applied recently to magnetic resonance spectroscopy (MRS) in solution, where it can be used to produce a large increase in sensitivity. Using this technique, the metabolism of several ^{13}C-labeled compounds has been observed and used to estimate rate constants for specific enzyme-catalyzed reactions *in vitro* and *in vivo* (Day SE, Kettunen

10   MI, Gallagher FA, Hu DE, Lerche M, Wolber J, Golman K, Ardenkjaer-Larsen JH, Brindle KM. Detecting tumor response to treatment using hyperpolarized ^{13}C magnetic resonance imaging and spectroscopy. Nat Med 2007;13:1382–1387; Gallagher FA, Kettunen MI, Hu DE, Jensen PR, Zandt RI, Karlsson M, Gisselsson A, Nelson SK, Witney TH, Bohndiek SE, Hansson G, Peitersen T, Lerche MH, Brindle KM. Production of hyperpolarized $[1,4-^{13}C_2]$malate from $[1,4-^{13}C_2]$fumarate is a marker of

15   cell necrosis and treatment response in tumors. Proc Natl Acad Sci USA 2009; 106:19801–19806). Furthermore, for some hyperpolarized ^{13}C-labeled substrates there is sufficient signal for the spatial distribution of both the substrate and its metabolites to be imaged *in vivo*. As some of these substrates have already been administered at relatively high concentrations in the clinic, this technique has the potential to be translated into clinical applications. To date, the most studied reactions have been

20   those involving hyperpolarized [1-^{13}C]pyruvate: the hyperpolarized label can be exchanged with either endogenous lactate or alanine, or alternatively it can be irreversibly converted to carbon dioxide, which is subsequently converted to bicarbonate in the reaction catalyzed by carbonic anhydrase. These metabolic reactions have been observed in tumors, in cardiac tissue and in the liver (Merritt ME, Harrison C, Storey C, Jeffrey FM, Sherry AD, Malloy CR. Hyperpolarized ^{13}C allows a direct measure

25   of flux through a single enzyme-catalyzed step by NMR. Proc Natl Acad Sci USA 2007;104:19773– 19777; Schroeder MA, Swietach P, Atherton HJ, Gallagher FA, Lee P, Radda GK, Clarke K, Tyler DJ. Measuring intracellular pH in the heart using hyperpolarized carbon dioxide and bicarbonate: a ^{13}C and ^{31}P MRS study. Cardiovasc Res 2010;86:82–91; Hu S, Chen AP, Zierhut ML, Bok R, Yen YF, Schroeder MA, Hurd RE, Nelson SJ, Kurhanewicz J, Vigneron DB. In vivo carbon-13 dynamic MRS

30   and MRSI of normal and fasted rat liver with hyperpolarized ^{13}C-pyruvate. Mol Imaging Biol 2009;11:399–407).

Recently, other endogenous molecules have been successfully hyperpolarized: tumor pH has been measured *in vivo* from the relative concentrations of ^{13}C-labeled bicarbonate and carbon dioxide following the injection of hyperpolarized ^{13}C-labeled bicarbonate (Gallagher FA, Kettunen MI, Day SE,

35   Hu DE, Ardenkjaer-Larsen JH, Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, Brindle KM. Magnetic resonance imaging of pH in vivo using hyperpolarized ^{13}C-labelled bicarbonate. Nature 2008;453:940–943); elevated levels of hyperpolarized malate have been demonstrated in necrotic tumor tissue *in vivo* following the injection of hyperpolarized ^{13}C-labeled fumarate (Gallagher FA, Kettunen MI, Hu DE, Jensen PR, Zandt RI, Karlsson M, Gisselsson A, Nelson SK, Witney TH,

Bohndiek SE, Hansson G, Peitersen T, Lerche MH, Brindle KM. Production of hyperpolarized [1,4-^{13}C$_2$]malate from [1,4-^{13}C$_2$]fumarate is a marker of cell necrosis and treatment response in tumors. Proc Natl Acad Sci USA 2009; 106:19801–19806); the metabolism of glutamine to glutamate, catalyzed by the mitochondrial enzyme glutaminase, has been observed following administration of hyperpolarized ^{13}C-labeled glutamine to cells in vitro (Gallagher FA, Kettunen MI, Day SE, Lerche M, Brindle KM. ^{13}C MR spectroscopy measurements of glutaminase activity in human hepatocellular carcinoma cells using hyperpolarized ^{13}C-labeled glutamine. Magn Reson Med 2008;60:253–257); the organ-specific metabolism of hyperpolarized ^{13}C-labeled acetate to acetyl-CoA and acetyl carnitine has been observed in vivo (Jensen PR, Peitersen T, Karlsson M, In 't Zandt R, Gisselsson A, Hansson G, Meier S, Lerche MH. Tissue-specific short chain fatty acid metabolism and slow metabolic recovery after ischemia from hyperpolarized NMR in vivo. J Biol Chem 2009;284:36077–36082), and the metabolism of branched chain amino acids has been observed in tumors following the addition of hyperpolarized ^{13}C-labeled α-ketoisocaproate (Karlsson M, Jensen PR, In 't Zandt R, Gisselsson A, Hansson G, Duus JO, Meier S, Lerche MH. Imaging of branched chain amino acid metabolism in tumors with hyperpolarized ^{13}C ketoisocaproate. Int J Cancer 2010;127:729–736.10).

Although its etiology is lacking, cancer is phenomenologically well characterized as a molecular disease. Different kinds of cancers may have very different biochemical forms, however they can share general molecular features.

Early diagnosis of cancer continues to be given large attention since diagnosis at an early stage often increases the chances of a successful treatment. In fact, early diagnosing cancer and ensuring access to optimum treatment can lead to significant improvements in survival.

Early diagnosing of cancer could be achieved by taking advantage of a general molecular feature shared by different types of cancer cells and whose alteration in cancer can be early detected.

Carboxylesterases (CE, EC 3.1.1.1) are a family of enzymes catalysing the chemical conversion of an ester in an acid and an alcohol. A general reaction scheme is shown below:

Carboxylesterases are ubiquitously expressed in mammalian tissues. The many CE isoforms have been classified into 5 super families (CE 1-5) based on amino acid homologies. The CE1 enzymes are mainly localized to the liver, however they are also expressed in most other tissue types. A rat specific CE1 isoform is secreted from the liver to the blood in rats and mice and this iso-enzyme is correlated to a high level of hydrolase activity detected in rodents compared to humans (Yan, B. Dongfang Y., Bullock, P., Parkinson, A., Rat serum carboxylesterases, 1995, *JBC*, 32 (270): 19128-34; Rudakova, EV., Botneva NP., Makhaeva, GF. Comparative analysis of esterase activities of human, mouse and rat blood, 2011, *Bulletin of experimental biology and medicine,* 152(1): 73-75). The

other important isoform is the CE2 family, which is also expressed in the human liver (approx. 4 times less than CE1) as well as in most other tissues to a higher degree than CE1 (Talvar, S. The expression of human carboxylesterases in normal tissues and cancer cell lines (2008), Master thesis).

5   The expression of carboxylesterases decreases in cancer in both animal and human tissue. In particular, in hepatoma cells a 4 times decrease in the expression of carboxyl esterase has been measured compared to normal hepatocytes. Dependent on the isoform the expression is reported to be approx. 1.5 - 4 times higher in normal tissue than in the corresponding malignant tissue (Talvar, 2008).

       A number of studies have been reported on carboxylesterases in cancer cells.

10   The expression of carboxylesterase was reported as detectable in human cancer cells (HEPG2) and approx. 3-4 times lower than the expression of carboxyl esterase in normal human liver (hepatocytes) (Talvar, 2008). A patient study on non-tumor and tumor tissues from liver cancer (HCC) patients showed an almost 3 times decrease in the expression of carboxyl esterase in the tumor tissue (Na, K. et al., Human plasma carboxylesterase 1, a novel serologic biomarker candidate for

15   hepatocellular carcinoma (2009), Proteomics, 9: 3989-99).

       Another study showed that the carboxylesterase activity was significantly lower in colon cancer xenografts compared to the corresponding normal colon tissue in mice (Jansen et al., CPT-11 in human colon cancer cell lines and xenografts: characterization of cellular sensitivity determinants, 1997, Int. J. Cancer 70:335-40.)

20   A study has been reported on lung cancer patients where carboxylesterase activity is correlated to esterase expression in healthy and cancer lung tissue. In this study, they find that the activity correlates well with the expression, which is shown to be approx. 1.5 times higher in healthy tissue (Liewald F. et al, Intracellular pH, esterase activity and DNA measurements of human lung carcinomas by flow cytometry. 1990, Cytometry, 11: 341-48)

25   WO2012102773 discloses a method for the diagnosis and treatment of cancer, in particular breast cancer, by measuring the activity of the enzyme PMPMEase (human carboxylesterase 1). Said activity is measured in a biological sample by assaying the enzyme expression or enzymatic activity, in the last case through the measurement of the consumption of a substrate or the production of a product. It is only generally stated that the enzyme assay can be performed *in vivo*.

30   US8198038 discloses a screening method to distinguish healthy human beings from those with human liver cancer (hepatocellular carcinoma; HCC) comprising the steps of collecting human blood and detecting the presence of human liver carboxylesterase 1 (hCE1) in the plasma, wherein the level of hCE1 protein is increased more in the plasma of patients with HCC than in the plasma of healthy patients.

35   The conversion of an hyperpolarized ester, catalysed by carboxylesterase, generates hyperpolarized metabolic products whose MR signals are well distinguishable from each other and from the injected substrate. Cancer cells and healthy cells convert a hyperpolarized ester to a different

degree, leading to differences in said metabolic product signal amounts, therefore said difference between the signals in tumor and non-tumor cells can be exploited to identify cancer.

A common weakness of the endogenous molecules known in the prior art, when used as hyperpolarized markers, is the relatively fast vanishing of MR signals, of both the substrate itself and its relevant metabolites. DNP in fact, as well as all other hyperpolarization procedures, leads to a transient enhancement of the nuclear magnetic order, that allows the *in vivo* visualization of said moieties but, unfortunately, for a limited amount of time, in the order of 3 to 5 times the longitudinal relaxation time of the nuclei used as MR probe. Since MR imaging procedures require a certain amount of time in order for the final image to be reconstructed with acceptable sensitivity and resolution, markers with slower nuclear relaxation, with respect to the product used so far, are strongly desirable, also in view of inherent technical features of the imaging procedure, such as dilution (upon injection into patient) and metabolic conversion (time delay for achieving detectable concentrations of metabolites).

Therefore, in order to be able to register *in vivo* metabolic maps of tumour and healthy tissues, with superior sensitivity and resolution with respect to the state of the art, novel metabolic contrast agents with enhanced persistence of the hyperpolarized signal (i.e. slower nuclear longitudinal relaxation) are strongly needed

SUMMARY OF THE INVENTION

It has now been found that 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane is characterized by a ^{13}C longitudinal relaxation time significantly longer than any other known molecule used for metabolic imaging.

Said 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane can be polarized by DNP in order to achieve a ^{13}C signal that allows its detection *in vivo* over an extended acquisition window. The conversion of said hyperpolarized ester, catalysed by carboxylesterase, generates hyperpolarized metabolic products whose MR signals are well distinguishable from each other and from the injected substrate.

Cancer cells and healthy cells convert 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane to a different degree leading to differences in said metabolic product signal amounts, therefore said difference between the signals in tumor and non-tumor cells can be exploited to identify cancer.

Within the context of cancer diagnosis, said difference in the signals of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane metabolized by a carboxylesterase can be used for detecting the presence of a tumor, for evaluating the efficacy of an anti-cancer therapy and/or to determine a time evolution of a tumor.

It has also been found that the overall hyperpolarized signal of the metabolites deriving from 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane is far more intense than the hyperpolarized signal of the metabolites generating from [1-^{13}C]-pyruvate, the most used MR metabolic agent, in particular in some cancer cell types.

Therefore, an object of the present invention is the compound 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane of formula (I):
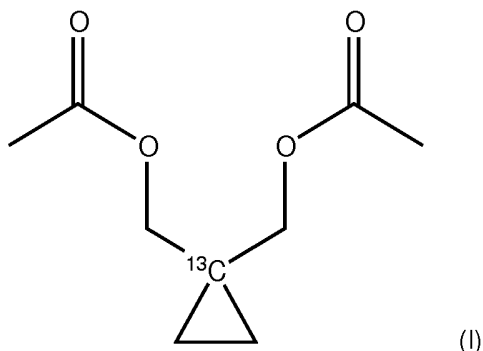
(I)

said compound being enriched above natural abundance of ^{13}C in position 1 of the molecule.

In a preferred embodiment, one or more hydrogen atoms of said 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane are replaced by deuterium. More preferably, all the methylene groups of the compound of formula (I) are deuterated, the comupund being thus identified as 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane and having the following structural formula (II):

(II)
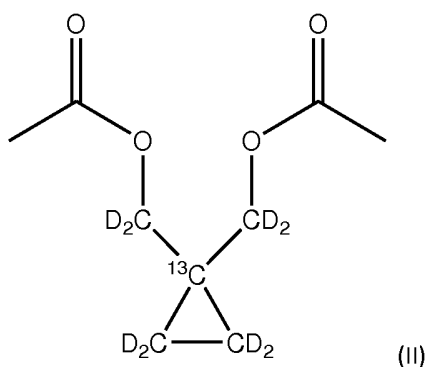
A process for the preparation of the new compound 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane comprising the steps of:

a) reacting 1-^{13}C-1,1-Bis(hydroxy(methyl))-2,2'-cyclopropane with acetyl chloride, and
b) removing the excess of acetyl chloride and the formed hydrochloric gas,

is also an object of the present invention.

The same process as above can be used for the preparation of deuterated 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane by using in step a) deuterated 1-^{13}C-1,1-Bis(hydroxy(methyl))-2,2'-cyclopropane.

The new compound can be hyperpolarized and used as a contrast agent in [13]C Magnetic Resonance diagnostic technique (^{13}C-MR) for the diagnosis of tumor.

It is also an object of the present invention a method of ^{13}C-MR detection of tumors using an imaging medium comprising hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane,

wherein signals of the corresponding hyperpolarized metabolic products of the carboxylesterase conversion are detected.

Said imaging medium comprising the hyperpolarized compound of the invention is also an object of the invention.

5   In a preferred embodiment of the present invention, the hyperpolarized metabolic products of carboxylesterase conversion of the compound of the invention are $1-^{13}C-1-(acetoxy(methyl))-1-(hydroxy(methyl))-2,2'-cyclopropane$ and/or $1-^{13}C-1,1-(dihydroxy(methyl))-2,2'-cyclopropane$ or a mixture thereof.

In a preferred embodiment, wherein the $1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane$ is
10   deuterated, its metabolic products are also deuterated and they are indicated in the following description as $1-^{13}C-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane$ and $1-^{13}C-1,1-(dihydroxy(methyl-d_2))-2,2'-d_4-cyclopropane$.

With respect to known hyperpolarized molecules used as metabolic markers, $1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane$ and its metabolic products $1-^{13}C-1-(acetoxy(methyl))-1-$
15   $(hydroxy(methyl))-2,2'-cyclopropane$ and $1-^{13}C-1,1-(dihydroxy(methyl))-2,2'-cyclopropane$ have a much slower ^{13}C MR signal decay, because of the unique quaternary position of the observed ^{13}C nuclei which minimizes any longitudinal relaxation effect due to dipolar interaction and chemical shift anisotropy. This is a great advantage for metabolic imaging based on hyperpolarized markers, that reflects into more pronounced metabolite signals as well as on a broader time window for signal
20   detection.

In an embodiment of the present invention, in the method of ^{13}C-MR detection a first signal obtained from a region of interest is compared with a second signal (typically a signal derived from a reference sample, e.g. a signal obtained from a corresponding non-tumor/healthy tissue); said comparison is useful to determine a difference between tumor and non-tumor tissue. Said comparison
25   can be used, for example, for the diagnosis of a tumor or to provide a localization of a tumor.

Furthermore, when a first signal obtained from a region of interest comprising a tumor tissue is compared with a second signal obtained from the same region of interest at an earlier time, the comparison between said first and said second signal can provide information about the the tumor development over time, which can also be an indication of aggressiveness, of the tumor and/or the
30   efficacy of a therapy when treating said tumor by (immune)pharmacological and/or surgical and/or radio therapy.

In a preferred embodiment of the invention, said first signal is the ratio between the signal of the metabolic product of the carboxylesterase conversion and the signal of the administered substrate (the hyperpolarized $1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane$) detected in the region of interest
35   and said second signal is the same ratio but detected in a reference sample, for example a corresponding non-tumor/healthy tissue, or in the same region at an earlier time.

Another object of the present invention is the above method of ^{13}C-MR detection wherein said signals are used to generate a metabolic profile, based on the metabolic carboxylesterase conversion of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane into the corresponding hyperpolarized metabolic products, wherein said metabolic profile is useful in detecting or providing

5   indication of a tumor.

In an embodiment of the present invention, said metabolic profile is determined in a region of interest (where the presence of a tumor tissue is known or suspected) and compared with a metabolic profile of reference (e.g. relative to a corresponding non-tumor tissue, typically a healthy tissue in the close proximity of the tumor tissue).

10   Another object of the present invention is a method for operating an MRI system comprising the steps of:

        a.   submitting a subject who is affected or suspected to be affected by a tumor, who has
        been positioned in an MRI system and treated with hyperpolarized 1-^{13}C-1,1-
        Bis(acetoxy(methyl))-2,2'-cyclopropane, and wherein said hyperpolarized ^{13}C ester has been
15   metabolically converted into the corresponding hyperpolarized metabolic product(s) of the
        carboxylesterase conversion, to a radio frequency pulse having a frequency selected to excite
        nuclear spin transitions in ^{13}C nuclei;

        b.   recording an MR signal from said excited nuclei; and

        c.   comparing a first MR signal deriving from a region of interest comprising said tumor or
20   said suspected tumor with a second MR signal deriving from said subject or from a sample taken
        from said subject.

In an embodiment of the invention, said second signal is an MR-signal deriving from a non-tumor tissue of said subject. In another embodiment of the invention, said second signal is an MR-signal which has been detected from the region of interest, at an earlier time with respect to the first signal.

25   Another object of the invention is the above method further comprising the steps of:

        d. determining a difference between said first signal and second signal;

        e. comparing said difference of step d) with a reference value, to produce a
        deviation value; and

        f. determining if the deviation value is, in absolute value, higher than a
30   predetermined value.

Another object of the present invention is the above method, wherein said second signal is determined on a non-tumor tissue, further comprising the step of:

        g. providing an indication of possible tumor affection in case the deviation value
        is in absolute value higher than said predetermined value.

Another object of the present invention is the above method for operating an MRI system comprising steps a) to f), wherein said second signal is determined in the region of interest, at an earlier moment in time with respect to the first signal, and optionally stored in the system, said method further comprising the step of:

5   g'.   providing an indication of tumor variation in case the deviation is in absolute value higher than said predetermined value.

Another object of the present invention is the above method for operating an MRI system comprising steps a) to f), wherein said subject has undergone an anti-tumor treatment and wherein said second signal is determined in the region of interest, at an earlier moment in time with respect to

10 said first signal, and optionally stored in the system, said method further comprising the step of:

         g".   providing an indication of efficacy of said treatment if this deviation is in absolute value higher than a predetermined value.

In a preferred embodiment, said second signal is determined before, after or at the beginning of the treatment, wherein the effective time of detection of said second signal will be decided by the

15 person skilled in the art, according to patient's conditions, kind of treatment, degree of severity of the disease and any other clinical parameter within the general knowledge on the matter. Examples of the time of determination of said second signal are few days, e.g. 1 to 5, one or more weeks, one or more months.

An MR system performing any of the methods above described is also an object of the present

20 invention.

The use of said MR system for providing an indication of the presence of a tumor, of its grade of aggressiveness or for monitoring the response to an antitumor therapy of a subject affected by a tumor is also within the scope of the present invention.

The present invention provides the advantages of making available an imaging medium

25 comprising hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane, which can be used in MRI technique for the diagnosis of tumors with a selective grade of distinction between tumor and non-tumor tissue.

A further advantage is represented by the possibility of taking different registrations of the MR signals of the hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane in a tumor tissue, while

30 an antitumor therapy is administered and to monitor the progress of the therapy.

A further advantage is represented by the possibility of detecting aggressive forms of tumors by monitoring the development of the formation of the corresponding ^{13}C hyperpolarized metabolic product(s) of the carboxylesterase conversion in a tumor tissue.

These and other objects of the present invention and advantages will be disclosed in detail in the

35 following description even by means of Figures and Examples.

Figures

9

Figure 1. Dissolution spectrum of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane.

Figure 2. In cell DNP conversion of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane and 1-^{13}C pyruvate in rat liver cancer cells (rat hepatoma, Morris). The DNP experiments were performed with 10 million cells. A) Build-up of the metabolites 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and 1-^{13}C-1,1-(dihydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane from injection of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and of 1-^{13}C-lactate from injection of 1-^{13}C-pyruvate, respectively into whole Morris cells. B) Area under the curve of the metabolites 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane and 1-^{13}C-lactate.

Figure 3. In cell DNP conversion of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and 1-^{13}C pyruvate in human prostate cancer cells (human prostate carcinoma, PC-3). The DNP experiments were performed with 10 million cells. A) Build-up of the metabolites 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane from injection of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and 1-^{13}C-lactate from injection of 1-^{13}C-pyruvate, respectively into whole PC-3 cells. B) Area under the curve of the metabolites 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and 1-^{13}C-lactate.

Figure 4. In cell DNP conversion of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane in human prostate cancer cells (human prostate carcinoma, PC-3) and in human prostate healthy cells (Immortalized human prostate cells, PNT-1A)). The DNP experiments were performed with 10 million cells. A) Build-up of the metabolite 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane in PC-3 cells (filled squares) and in PNT-1A cells (open circles). B) Area under the curve of the metabolite 1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane produced in PC-3 cells and in PNT-1A cells.

Figure 5. ^{13}C NMR sum spectrum, obtained by integrating over time the spectra of a time series acquired on the prostate of a representative animal. 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane is labelled as A, whereas the metabolites signals are labelled as B (1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane) and C (1-^{13}C-1,1-Bis(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane).

Figure 6. Time course of the ^{13}C NMR signals in rat prostates (average over n = 2 subjects) after injection of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane (solid line). The evolution of the metabolites signals is represented by a wide dashed line (1-^{13}C-1-(acetoxy(methyl-d$_2$))-1-(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane) and by a narrow dashed line (1-^{13}C-1,1-Bis(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane) respectively.

DETAILED DESCRIPTION OF THE INVENTION

Within the scopes of the present invention, the term MRI means Imaging (typically for diagnostic purposes) by means of Magnetic Resonance (MR) as commonly intended in the state of the art and for example disclosed in WO200977575 and the references cited therein.

Within the scopes of the present invention, the "imaging medium" and "contrast agent" are used synonymously, as commonly intended in the state of the art and for example disclosed in WO200977575 and the references cited therein.

Within the scopes of the present invention, the terms "hyperpolarization", "hyperpolarized" or similar mean enhancing the nuclear polarization of NMR active nuclei present in the high T_1 agent as commonly intended in the state of the art and for example disclosed in WO200977575 and the references cited therein.

Within the scopes of the present invention, the term Dynamic Nuclear Polarization (DNP) is a technique in Magnetic Resonance Imaging as commonly intended in the state of the art and for example disclosed in WO200977575 and the references cited therein.

Within the meaning of the present invention, the term "hyperpolarized" means the nuclear spin polarization of a compound higher than thermal equilibrium.
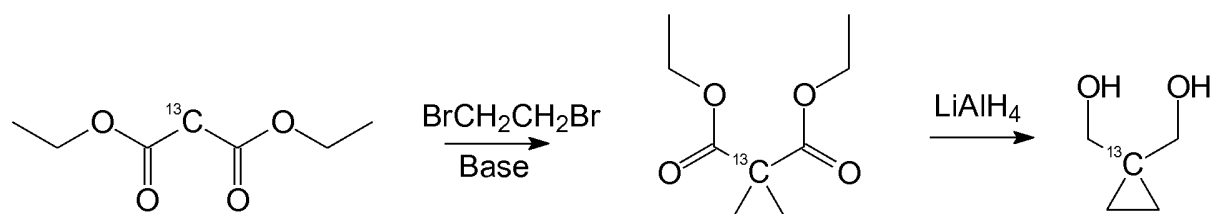
Within the scope of the present invention "MRI system" means apparatus, equipment and all features and accessories useful for performing MR experiments, in particular for diagnostic purposes.

Within the meaning of the present invention, "1-^{13}C" means that the labeled compound is enriched in ^{13}C in position 1 of the molecule. The term "enriched" means that the concentration of the non-zero nuclear spin nuclei in the compound (in particular of ^{13}C in position 1) is above the typical value of natural abundance of said nuclei, preferably above at least 10% of natural abundance, more preferably above at least 25%, and even more preferably above at least 75% of its natural abundance and most preferably above at least 90% of its natural abundance. Enrichment can be achieved by chemical synthesis or biological labeling, according to the prior art teachings. Enrichment of non-zero nuclear spin nuclei over natural abundance may be determined, for instance, on a reference amount of the material, e.g. at least 0.1 mmole, preferably at least 1 mmole of the material.

The new compound 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane is synthetized starting from 1-^{13}C-1,1-Bis(hydroxy(methyl))-2,2'-cyclopropane according to methods known in the art, typically by esterifying the alcohol groups of the starting compound and isolating the final compound by any conventional means known in the art. For instance, acetyl chloride is added, preferably in excess, to the starting compound and the resulting mixture is stirred to obtain the desired product 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane. Preferably, the excess of acetyl chloride and the formed hydrochloric gas are removed from the mixture, and the final compound is isolated and recovered from the mixture.

The starting compound 1-^{13}C-1,1-Bis(hydroxy(methyl))-2,2'-cyclopropane can be obtained according to any preparation methods known in the art, starting from commercially available ^{13}C-labelled diethyl malonate.

For instance, ^{13}C-labelled diethyl malonate can be subjected to a double alkylation by reacting it with 1,2-dibromethane and subsequent reduction of the ester groups with LiAlH$_4$, according to the following reaction scheme:

11

$$\text{diethyl malonate (1-}^{13}\text{C)} \xrightarrow[\text{Base}]{\text{BrCH}_2\text{CH}_2\text{Br}} \text{cyclopropane diester (}^{13}\text{C)} \xrightarrow{\text{LiAlH}_4} \text{1-}^{13}\text{C-1,1-bis(hydroxymethyl)cyclopropane (OH, OH)}$$

as described by House, H.O. et al.. "The synthesis of spiropentane-d8". J. Org. Chem. 1956, 21,1487–149. Alternatively, diethyl malonate can be reacted with dihaloethane to provide a malonic acid derivative with a cyclopropyl group in 2-position, as described for instance by Singh R. K. and Danishefsky S., J. Org. Chem. 1975, 40(20), 2969-2970. The malonic acid derivative is then reduced as above described with LiAlH$_4$ to give the desired diol compound.

In a preferred embodiment of the process, the starting compound is deuterated 1-13C-1,1-Bis(hydroxy(methyl-d2))-2,2'-d4-cyclopropane which can be obtained as described above by reacting from a corresponding commercially available 13C-labelled diethyl malonate with respective deuterated reactants, i.e. BrCD2CD2Br and LiAlD4, both commercially available; according to the preparation methods described above (where the hydrogen atoms of the respective reactants are replaced by deuterium); the obtained product of the invention, 1-13C-1,1-Bis(acetoxy(methyl-d2))-2,2'-d4-cyclopropane, is thus also deuterated.

The 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane is hyperpolarized by Dynamic Nuclear Polarization (DNP), which is a known method disclosed, for example, in WO9935508, and in particular in WO2011124672. Hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane is obtained. It can be used in an imaging medium in a method of ^{13}C-MR detection.

The activity of the carboxylesterase isoforms CE1 and CE2 is highly substrate dependent. In general substrates with a smaller alcohol group than acid group are reported to have higher affinity for the carboxylesterase isoform CE1 and the reverse class of substrates with a larger alcohol group than acid group have higher affinity for the CE2 enzyme (Imai, T. Human Carboxylesterase Isozymes: Catalytic Properties and Rational Drug Design, (2006) Drug Metab. Pharmacokinet 21(3): 173-85.).

The diacetate ester of the invention, 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane, is a substrate for the CE2 isoform.

It provides the advantage of being effectively hydrolyzed in liver, prostate and breast cells, where the CE2 enzyme is highly expressed. Especially in prostate cells, where the CE2 expression is high.

1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane also provides good chemical and physical properties, as high solubility, high polarization, very long T1 (as compared to other compounds employed for metabolic imaging, illustrated in the following table 1), sufficient chemical shift separation between substrate and product to detect the hydrolysis product *in vivo*.

Table 1: T1 of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane compared with other metabolic imaging compounds

Compound	T1, 37 C, 14.1 T
1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4- cyclopropane	85 ± 4
1-13C-pyruvate	45 ± 4
1-13C-acetate	50 ± 3
1-13C-lactate	40 ± 3
2-13C-1,1,2,2-d4-choline	43 ± 4
1,3-ethyl acetoacetate	27 ± 2
13C6-glucose	15 ± 2
1-13C-glucose	20 ± 2
1-13C-2-ketoisocaproate	35 ± 4
1-13C-alanine	31 ± 2
5-13C-glutamine	22 ± 2
1,4-13C2-Fumarate	35 ± 3
1,4-13C2-malate	30 ± 3
1-13C-bicarbonate	30 ± 2
1-13C-2-oxoglutarate	30 ± 3

A further advantage of the use of the compound of the invention as a metabolic substrate is that its uptake into cells takes place mainly by diffusion through the cell membrane. Therefore, it is not uptake-limited and only the activity of the metabolizing enzyme itself has influence on the amount of hyperpolarized product that is produced. This means that the detected signal in the present invention is highly representative of the activity of the carboxylesterase, thus making said substrate particularly useful as real time molecular contrast agents. On the contrary, substrates like mono-carboxylic acids, e.g. pyruvic acid, can suffer the disadvantage of being uptake-limited; the signal of their hyperpolarized product may therefore be not representative of the activity of the specific enzyme to be detected.

The method of the present invention is a non-invasive method, which allows a real time metabolic assessment of the carboxylesterase activity *in vivo*. An image representative of said activity is collected seconds to minutes following intravenous injection of the substrate.

Essentially, the method of operating an MRI system according to the present invention comprises the steps of a) recording an MR signal from the excited nuclei; and b) comparing a first MR signal deriving from the tumor or suspected tumor with a second MR signal deriving from the same subject or from a sample thereof.

In a preferred embodiment of the invention, said first signal deriving from said tumor is lower than said second MR signal.

In an embodiment of the present invention, as shown in steps d-f above, the MRI apparatus can process said first signal and said second signal by comparing each other, calculating a difference between the two signals and comparing said difference with a reference value; as shown in step g above, if this comparison provides a value which is, in absolute value, higher than a predetermined value, then said MRI apparatus provides an indication of possible tumor affection.

The use of said apparatus for monitoring the response of a subject affected by a tumor to antitumor therapy (step g') or for evaluating the aggressiveness of a tumor (step g") are further objects of the present invention.

Examples of said tumors are tumors selected from the group consisting of liver, colon, prostate
5   and breast. In a preferred embodiment, the tumor is prostate tumor.

According to the present invention, the compound $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl))\text{-}2,2'$-cyclopropane can be exploited as a marker of targeted therapies, where for targeted therapy is intended the targeting of molecules important for the carcinogenesis of the cancer cells.

In carrying out the methods of the present invention, the first signal (S_1), the second signal (S_2)
10   and the reference value (R), depend on how the methods of the invention are applied.

Typically, in order to have comparable data, the MR signals obtained in the method of the invention are normalized with respect to the corresponding signal of the $1\text{-}^{13}C\text{-}1,1$-Bis(acetoxy(methyl)-2,2'-cyclopropane.

When the method of the present invention is performed to provide an indication of possible tumor
15   affection, said first signal S_1 is the ratio between the integral of the MR line of the hyperpolarized metabolic product of the carboxylesterase conversion and the integral of the MR line of the administered substrate (the hyperpolarized $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl)\text{-}2,2'$-cyclopropane), detected in the region of interest comprising the alleged tumor, while the second signal S_2 is the analogue ratio calculated in non-tumor tissue; the reference value R is either equal to S_2 or, in case no
20   signal of the hyperpolarized metabolic product is detected in the healthy tissue under consideration, R is set to 3 times the noise standard deviation divided by the substrate signal in the same volume. Preferably, non-tumor tissue is surrounding the tumor, so that the MR system can provide an accurate imaging of the tumor, which is of great importance for the evaluation of surgical intervention.

In case the purpose of the method is a follow-up of antitumor therapy, said first signal
25   corresponds to the metabolic product signal detected in the tumor before, or at the start or at a certain point after the beginning of therapy and said second signal is the one produced by the same tumor after a certain period subsequent to the detection of said first signal. The reference value R is set equal to the first signal.

In case the purpose of the method is to determine aggressiveness of a tumor, said first signal is
30   the one produced by the tumor at the start of the determination and said second signal is the one produced by the same tumor after a certain period subsequent to the detection of said first signal. Again the reference value is set equal to the first signal.

The first and second MR signals can be obtained either as single signals or calculated as a mean value of a plurality of respective signals determined (from different voxels) in a selected region of
35   interest (S_1) or in a non-tumor tissue (S_2).

In an embodiment of the invention, said first signal and said second signal can be directly compared, either as single signals or as mean values of a plurality of signals, to obtain the desired information on the tumor tissue. In an alternative embodiment of the invention, the signals can be used to generate a parametric image and the comparison can be performed by comparing the zones of said image corresponding to said first and said second signal.

According to the present invention, a difference between said first and said second signal is determined. This difference (S_1-S_2) is important for the different scopes of the present invention.

This difference is compared with the reference value to produce a value representing the deviation (D) of said difference from said reference value:

$$D=(S_1-S_2)/R.$$

If it is determined that this deviation provides a value which is, in absolute value, higher than a predetermined value, this deviation provides an indication of possible tumor affection, of the efficacy of the antitumor therapy or of tumor aggressiveness, depending on the purpose of the method of the invention.

For instance, in an embodiment of the invention, said predetermined value can be set at 2; accordingly, if the calculated value "D" is equal or higher than 2, this can be indicative of a possible presence of a tumor in the region of interest, of the efficacy of the antitumor therapy or of tumor aggressiveness, depending on the purpose of the method of the invention. Preferably a deviation value D of from 2 to 10 can be indicative of said presence, efficacy or aggressiveness, more preferably a deviation from 2 to 20, even more preferably a deviation from 2 to 40, particularly preferred is a deviation from 2 to 60, maximally preferred is a deviation from 2 to 80, the most preferred is a deviation from 2 to 100 or higher.

In an embodiment of the invention, the method is performed on a subject who is suspected to suffer or suffers from a tumor.

In another embodiment of the present invention, the above method is performed on a subject who is undergoing or has been subjected to an antitumor treatment and the reference value is the signal of the hyperpolarized metabolic product(s) of the carboxylesterase conversion in said region of interest determined before, during or after said treatment. As above, if a deviation D is calculated which is higher, in absolute value, than a predetermined value (e.g. higher than 2, and preferably within the above indicated ranges), this provides an indication of the efficacy of the antitumor treatment.

In some embodiments, the present invention can be used in the field of so-called "personalized medicine", or similarly intended. As explained above, tumor therapy is affected by variations in its efficacy even on the same type of tumor and with the same anticancer therapeutic protocol. Such variations are due to the different individual responses by the patients. Carrying out the method of the present invention allows to monitor (follow-up) the efficacy of a tumor therapy and, in case, allowing the doctor to fit the therapy to the patient.

Typical metabolic imaging procedures with the compound of the invention in human subjects should be performed at magnetic fields $\geq$ 1 T. Field strengths of 1.5 T or higher are preferred since the spectral separation between the injected substrate (ester) and the observed metabolite (acid or alcohol) scales linearly with the intensity of the applied field. The MR scanner should be capable to

5   detect ^{13}C signals in addition to 1H and although not always mandatory, surface or endoscopic radiofrequency coils could allow achieving better results in specific organs. For prostate investigation for instance, an endorectal ^{13}C is expected to strongly increase the sensitivity of the method with respect to a standard whole body resonator. Being the hyperpolarized signals typically available for a time range in the order of 3 to 5 times the longitudinal relaxation rate of 1-^{13}C-1,1-

10 Bis(acetoxy(methyl))-2,2'-cyclopropane, the total acquisition time for a metabolic MR procedure will not exceed 5 min. Spectroscopic imaging sequences such as Single Voxel Spectroscopy (SVS) or Chemical Shift Imaging (CSI) need to be used in order to separate the signal coming from the substrate from that coming from the hyperpolarized metabolic product. Fast spectroscopic imaging sequences such as EPSI are preferred due to the limited time available for the acquisition.

15 In order for the method to provide enough sensitivity, 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane formulations and dissolution/transport protocols which allow to maintain at least 10% polarization at time of injection are preferred. Preferably, at least of about 20% polarization is maintained, more preferably at least of about 30% polarization is maintained, even more preferably at least of about 60% polarization is maintained, most preferably at least of about 80% polarization is

20 maintained. Examples of said dissolution/transport protocols are described, for instance, in WO 02/36005.

The present invention will be further illustrated by the following examples.

EXAMPLES

Where not otherwise specified, chemicals and reagents used in the following examples are

25 commercially available or can be prepared according to methods well-known in the art.

Example 1. Synthesis of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane

284 mg, 2.55 mmol of 1-^{13}C-1,1-Bis(hydroxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane, (prepared as described by House, H.O. et al.. "The synthesis of spiropentane-d8". J. Org. Chem. 1956, 21,1487–149) were put in a glass flask and cooled to 0 °C on an ice bath. Acetyl chloride (3 ml, 34 mmol) was

30 added slowly while stirring. After complete addition the mixture was allowed to warm to room temperature and stirred for additionally 12 h. The excess acetyl chloride and the formed hydrochloric gas were then removed in vacuum. The compound of formula (II) 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane was recovered as a colorless oil; yield: 445 mg (2.27 mmol, 90%).

Spectral data are consistent with the expected structure, as illustrated below:
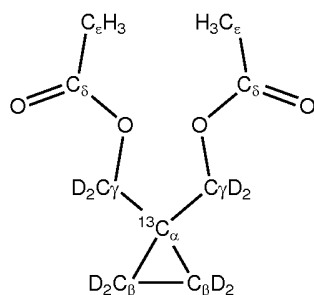
^{1}H NMR (Acetone-d$_6$, ppm): 2.01 (singlet)

^{13}C NMR (D$_2$O, ppm): 9.4 (multiplet, β), 19.6 (singlet, α, ^{13}C label), 21.4 (singlet, ε), 70.1 (multiplet, γ), 175.8 (singlet, δ)

Example 2. Preparation of Hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane

A) Finland radical, carboxylic acid form (0.7 mg, 0.67 μmol) was dissolved in 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane (45 μl, 50.7 mg, 0.27 mmol). To the solution was added a DMSO solution of the gadolinium complex ([alfa1,alfa4,alfa7-tris[(phenylmethoxy)methyl]-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetato(4-)] gadolinate(1-)]hydrogen) (0.75 mg of a 100 μmol / g solution). The concentration of radical and gadolinium were 15 mM and 1.6 mM respectively.

B) 30 μmol of a 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane sample made following the description in example 2.A was hyperpolarized. The composition was hyperpolarised under DNP conditions at 1.2 K in a 3.35 T magnetic field under irradiation with microwave (93.900 GHz). The polarization build-up constant was 750 s. The solid-state polarization was approx. 15%.

C) The sample was dissolved in 5ml phosphate buffer (40 mM, pH 7.3). The pH after dissolution was 7.3. The solution was collected directly into a 10 mm NMR tube and transferred to a 14.1 T magnet (pH 7.2) where a time series of 5 degree 1D ^{13}C-NMR spectra were recorded with a total delay between the pulses of 3 s. The liquid state polarization was 13 % (12 s after dissolution) and the liquid state T$_1$ was approx. 85 s at 14.1 T and 37 C. The 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane was not hydrolysed in the dissolution process, Figure 1.

Example 3 - Comparison between metabolism of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$-cyclopropane and hyperpolarized 1-^{13}C-pyruvate in rat liver (Morris7777)

Materials and methods

The experiments were performed with a co-polarization of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane and 1-^{13}C-pyruvic acid in equal amounts of compounds (30 μmol) resulting in a concentration of approx. 3.5 mM of each substrate in the experiments. The DNP preparation of 1-^{13}C-1,1-Bis(acetoxy(methyl-d$_2$))-2,2'-d$_4$- cyclopropane was performed as described in example 2 and the DNP preparation of 1-^{13}C-pyruvate was performed as described in WO 2006/011809. The two substrates were co-polarized without mixing the substrates.

Rat liver cancer cells (Morris7777) were grown in RPMI + 10% FBS and antibiotics. Following trypsin harvesting 10 million cells were redissolved in 500 µl phosphate buffer (PBS) and transferred to a 10 mm NMR tube and placed with connecting tubing in a 14.1 T magnet at 37 C.

5   Following dissolution in 5 ml phosphate buffer (40 mM pH 7.3) with addition of 2.5 µl NaOH to neutralize the pyruvic acid 2 ml of the substrate mixture was injected into 10 million cells in suspension. A series of 20 degree pulses every 2 s (56 scans in total) was acquired. The acquisition was started just before injection of the hyperpolarized substrate. Data are presented as metabolite signals as a function of time or as area under the curve of the metabolite signals.

Results

10   An account of produced hyperpolarized $1\text{-}^{13}C\text{-}1\text{-}(acetoxy(methyl-d_2))\text{-}1\text{-}(hydroxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-}$ cyclopropane and $1\text{-}^{13}C\text{-lactate}$ in rat liver cancer cells are shown in Figure 2 and Table 2.

Metabolite	AUC (arb. Units)
$1\text{-}^{13}C\text{-}1\text{-}(acetoxy(methyl-d_2))\text{-}1\text{-}(hydroxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-}$ cyclopropane	43480
$1\text{-}^{13}C\text{-}1,1\text{-}(dihydroxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-}$ cyclopropane	1559
$1\text{-}^{13}C\text{-lactate}$	282

Table 2. Area under the metabolic curves for the two metabolites arising from $1\text{-}^{13}C\text{-}1,1\text{-}$Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane and from $1\text{-}^{13}C\text{-lactate}$ in Morris7777 cells.

It can be seen from this example that it is possible to follow the build-up of both the mono ester,
15   $1\text{-}^{13}C\text{-}1\text{-}(acetoxy(methyl-d_2))\text{-}1\text{-}(hydroxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-cyclopropane}$ and $1\text{-}^{13}C\text{-}1,1\text{-}$(dihydroxy(methyl-d_2))-2,2'-d_4-cyclopropane resulting from the hydrolysis of hyperpolarized $1\text{-}^{13}C\text{-}1,1\text{-}$Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in whole Morris7777 cells. It can also be appreciated that the metabolic conversion of hyperpolarized $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-cyclopropane}$ is approx. 15 times higher than that of hyperpolarized $1\text{-}^{13}C$ pyruvate when comparing the maximum
20   metabolite signal. Due to the very long T_1 of $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-cyclopropane}$ the signal area under the curve of the mono-ester metabolite is significantly larger than the area under the curve of $1\text{-}^{13}C\text{-lactate}$. This enables a potentially high quality image of the in vivo signal with the $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl-d_2))\text{-}2,2'\text{-}d_4\text{-cyclopropane}$ metabolite exploring an AUC which is almost 30 times that of $1\text{-}^{13}C\text{-lactate}$.

25   **Example 4. Carboxyl esterase CE-2 activities measured with hyperpolarized $1\text{-}^{13}C\text{-}1,1\text{-}$Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in human prostate cancer cells (PC-3) in comparison to hyperpolarized $1\text{-}^{13}C\text{-pyruvate}$**

Materials and methods

The experiments were performed with a co-polarization of $1\text{-}^{13}C\text{-}1,1\text{-}Bis(acetoxy(methyl-d_2))\text{-}2,2'\text{-}$
30   d_4- cyclopropane and $1\text{-}^{13}C\text{-pyruvic}$ acid in equal amounts of compounds (30 µmol) resulting in a

concentration of approx. 3.5 mM of each substrate in the experiments. The DNP preparation of $1\text{-}^{13}\text{C}\text{-}$ 1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane was performed as described in example 2 and the DNP preparation of $1\text{-}^{13}\text{C}$-pyruvate was performed as described in WO 2006/011809. The two substrates were co-polarized without mixing the substrates.

5   Human cancer cells (PC-3) were grown in RPMI + 10% FBS and antibiotics. Following trypsin harvesting 10 million cells were redissolved in 500 μl phosphate buffer (PBS) and transferred to a 10 mm NMR tube and placed with connecting tubing in a 14.1 T magnet at 37°C.

Following dissolution in 5 ml phosphate buffer (40 mM pH 7.3) with addition of 2.5 μl NaOH to neutralize the pyruvic acid 2 ml of the substrate mixture was injected into 10 million cells in
10   suspension. A series of 20 degree pulses every 2 s (56 scans in total) was acquired. The acquisition was started just before injection of the hyperpolarized substrate. Data are presented as metabolite signals as a function of time or as area under the curve of the metabolite signals.

Results

An account of produced hyperpolarized $1\text{-}^{13}\text{C}$-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-
15   d_4- cyclopropane and $1\text{-}^{13}\text{C}$-lactate in human prostate cancer cells are shown in Figure 3 and Table 3.

Metabolite	AUC (arb. Units)
$1\text{-}^{13}\text{C}$-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane	20550
$1\text{-}^{13}\text{C}$-lactate	3766

Table 3. Area under the metabolic curves for $1\text{-}^{13}\text{C}$-1-(acetoxy(methyl-d_2))- 1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane and $1\text{-}^{13}\text{C}$-lactate in PC-3 cells.

It can be seen from this example that it is possible to follow the build-up of the mono ester, $1\text{-}^{13}\text{C}$-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane resulting from the hydrolysis of
20   hyperpolarized $1\text{-}^{13}\text{C}$-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in whole PC-3 cells. It can also be appreciated that the metabolic conversion of hyperpolarized $1\text{-}^{13}\text{C}$-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane is more than 3 times higher than that of hyperpolarized $1\text{-}^{13}\text{C}$ pyruvate when comparing the maximum metabolite signal. Due to the very long T_1 of $1\text{-}^{13}\text{C}$-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane the signal area under the curve of the mono-ester metabolite is significantly
25   longer than the area under the curve of $1\text{-}^{13}\text{C}$-lactate. This enables a potentially high quality image of the *in vivo* signal with the $1\text{-}^{13}\text{C}$-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane metabolite exploring an AUC which is almost 6 times that of $1\text{-}^{13}\text{C}$-lactate.

Example 5. Carboxyl esterase CE-2 activities measured with hyperpolarized $1\text{-}^{13}\text{C}$-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in human prostate cancer cells (PC-3) in
30   **comparison to healthy human prostate cells (PNT-1A)**

Materials and methods

The experiments were performed with a polarization of 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane (30 μmol) resulting in a concentration of approx. 3.5 mM of this substrate in the experiments. The DNP preparation of 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane was performed as described in example 2.

5   Human prostate cancer cells (PC-3) or immortalized human prostate healthy cells were grown in RPMI + 10% FBS and antibiotics. Following trypsin harvesting 10 million cells were redissolved in 500 μl phosphate buffer (PBS) and transferred to a 10 mm NMR tube and placed with connecting tubing in a 14.1 T magnet at 37°C.

Following dissolution in 5 ml phosphate buffer (40 mM pH 7.3), 2 ml of the substrate mixture was
10   injected into 10 million cells in suspension. A series of 20 degree pulses every 2 s (56 scans in total) was acquired. The acquisition was started just before injection of the hyperpolarized substrate. Data are presented as metabolite signals as a function of time or as area under the curve of the metabolite signals.

Results

15   An account of produced hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in prostate cancer and prostate healthy cells is shown in Figure 4.

It can be seen from this example that it is possible to follow the build-up of the mono ester, 1-^{13}C-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane resulting from the hydrolysis of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane in human prostate cancer PC-3
20   cells and in human healthy prostate cells. It can also be appreciated that the metabolic conversion of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane is approx. 6 times higher in the diseased cells than in the healthy cells. This difference suggests that a large contrast between diseased and healthy tissue can be expected in a human cancerous prostate.

Example 6: Conversion of hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-
25   **cyclopropane into hyperpolarized 1-^{13}C-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-**
cyclopropane and 1-^{13}C-1,1-Bis(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane in the healthy
prostate of living rats.

Materials and methods

DNP-MRI experiment has been performed on 2 healthy Copenhagen rats, 9 weeks old, with
30   average weight of 180 g.

0.24 mmol of a 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane sample prepared following the description in Example 2.A was hyperpolarized according to the conditions reported in example 2.B. The solid sample was then dissolved in 5 ml TRIS buffer (100 mM, pH 7.7) to obtain a hyperpolarized solution with 48 mM substrate concentration and a pH of 7.

35   2.8 ml of the dissolved hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane solution was injected intravenously at an injection rate of about 0.25 mL/s through a catheter placed in

the tail vein of the animal, resulting in a total administered dose of about 0.4 mmol/kg. A time series of 64 NMR spectra separated by 3 s and generated by 10° radiofrequency pulses was acquired starting from 15 s before injection of the hyperpolarized substrate, in order to follow the whole metabolic fate of the molecules and the decay of the hyperpolarized signal. The ^{13}C MR signal was collected by a 20

5   mm surface coil placed around the prostate. Spatial localization of the signal has been achieved by combining the limited sensitivity volume of the receiving coil with a slice selective spectroscopic sequence including a single gradient kept on only during the excitation period.

Results

Hyperpolarized 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane is taken up by prostate

10   cells and converted into its hyperpolarized metabolites, 1-^{13}C-1-(acetoxy(methyl-d_2))-1-(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane and 1-^{13}C-1,1-Bis(hydroxy(methyl-d_2))-2,2'-d_4-cyclopropane, by a two steps metabolic process clearly observable, *in vivo*, on the time scale of the DNP experiment. A ^{13}C NMR sum spectrum, obtained by integrating over time all the non-vanishing spectra of a time series acquired on a representative animal, is reported in Figure 5. The chemical shift differences

15   between the injected substrate and the relevant metabolic products are notable, allowing an unassailable identification of the different ^{13}C labelled species which are present in the tissue under investigation. The time evolution of the hyperpolarized ^{13}C signals of individual species is shown in Figure 6. While the signal of 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-d_4-cyclopropane is decaying, the products signal firstly build up, due to metabolic conversion of 1-^{13}C-1,1-Bis(acetoxy(methyl-d_2))-2,2'-

20   d_4-cyclopropane and then gradually decay according to their T_1 relaxation rate.
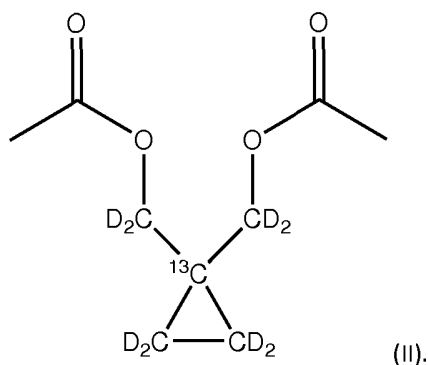
21

CLAIMS

1. The compound 1-^{13}C-1,1-Bis(acetoxy(methyl))-2,2'-cyclopropane, of formula (I)
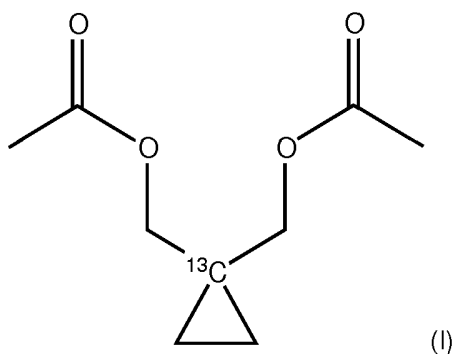
(I)

said compound being enriched above natural abundance of ^{13}C in position 1 of the molecule.

2. The deuterated compound of claim 1 of formula (II):

(II).

3. A compound according to claim 1 or 2 wherein said compound is hyperpolarized.

4. An imaging medium comprising the compound according to claim 3.

5. The compound according to any of claims 1, 2 or 3 for use as a contrast agent in ^{13}C Magnetic Resonance diagnostic *in-vivo* technique (^{13}C-MR).

6. The compound of claim 5 for use in the diagnosis of tumor.

7. Use of a compound according to claim 1 or 2 as contrast agent in *in-vitro* or *ex-vivo* ^{13}C Magnetic Resonance Imaging diagnostic technique (^{13}C-MRI).

8. Use according to claim 7 for the diagnosis of tumor.

9. A method of ^{13}C-MR detection of tumor using the imaging medium according to claim 4 wherein signals of the at least one hyperpolarized metabolic product of a carboxylesterase conversion of said hyperpolarized compound are detected.

10. The method according to claim 9, wherein said at least one hyperpolarized metabolic product is 1-^{13}C-1-(acetoxy(methyl))-1-(hydroxy(methyl))-2,2'-cyclopropane, 1-^{13}C-1,1-(dihydroxy(methyl))-2,2'-cyclopropane or a mixture thereof.

11. The method according to anyone of claims 9 or 10 wherein a first signal obtained from a region of interest is compared with a second signal and the comparison is used to determine a difference between tumor and non-tumor tissue.

12. A method for operating an MRI system comprising the steps of:
    a. submitting a subject who is affected or suspected to be affected by a tumor, who has been positioned in an MRI system and treated with the compound of claim 3, and wherein said compound has been metabolically converted into the corresponding hyperpolarized metabolic product(s) of a carboxylesterase conversion, to a radiation having a frequency selected to excite nuclear spin transitions in ^{13}C nuclei;
    b. recording an MR signal from said excited nuclei; and
    c. comparing a first MR signal deriving from a region of interest comprising said tumor or said suspected tumor with a second MR signal deriving from said subject or from a sample taken from said subject.

13. The method according to claim 12 further comprising the steps of:
    d. determining a difference between said first signal and second signal;
    e. comparing said difference of step d) with a reference value, to produce a deviation value; and
    f. determining if the deviation value is, in absolute value, higher than a predetermined value.

14. The method according to claim 13, wherein said second signal is determined on a non-tumor tissue, further comprising the step of:
    g. providing an indication of possible tumor affection in case the deviation value is in absolute value higher than said predetermined value.

15. The method according to claim 13, wherein said second signal is determined in the region of interest, at an earlier moment in time with respect to the first signal, and optionally stored in the system, said method further comprising the step of:

g'. providing an indication of tumor variation in case the deviation is in absolute value higher than said predetermined value.

16. The method according to claim 12, wherein said subject has undergone an anti-tumor treatment and wherein said second signal is determined in the region of interest, at an earlier moment in time with respect to said first signal, and optionally stored in the system, said method further comprising the step of:

g". providing an indication of efficacy of said treatment if this deviation is in absolute value higher than a predetermined value.

17. The method according to claim 16 wherein said second signal is determined before, after or at the beginning of the treatment.

18. A process for the preparation of the compound of claims 1 or 2 comprising the steps of:

a) reacting 1-^{13}C-1,1-Bis(hydroxy(methyl))-2,2'-cyclopropane, or its corresponding deuterated analog, with acetyl chloride, and

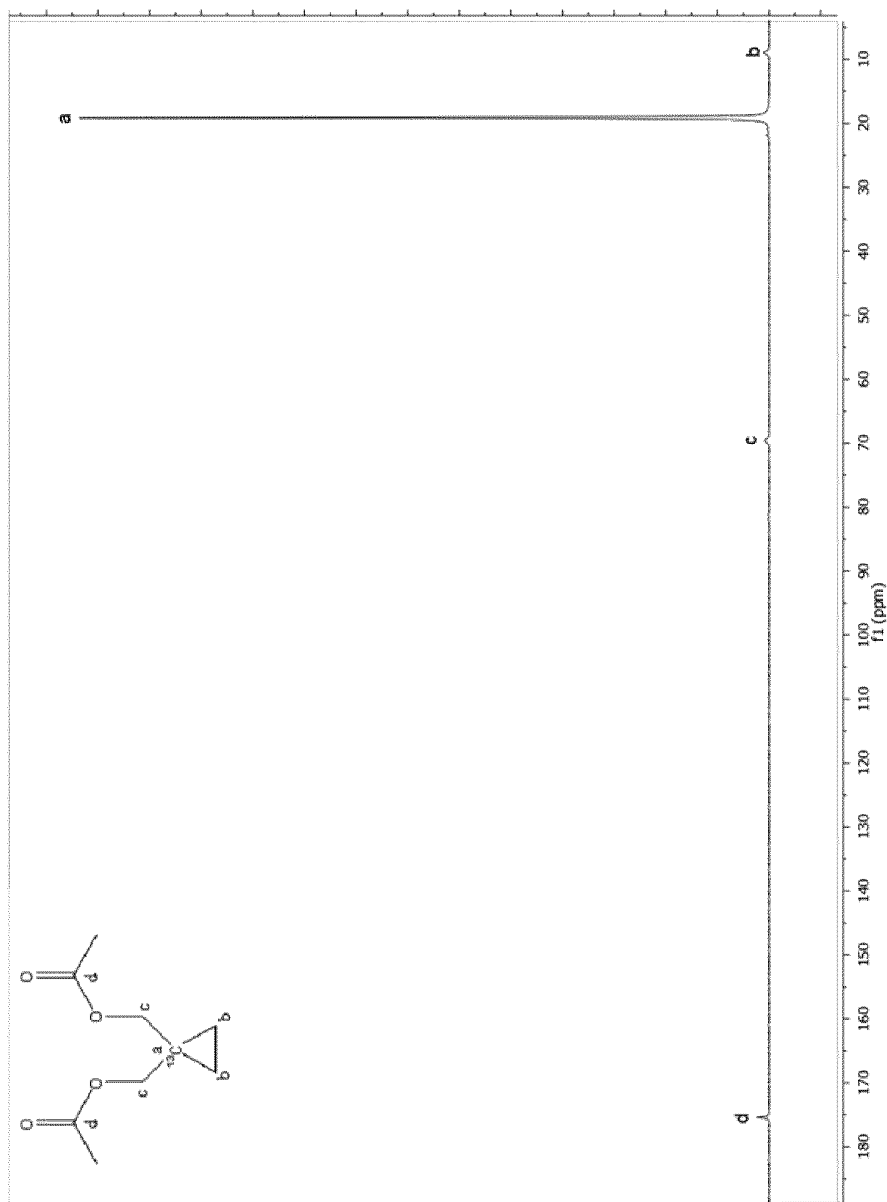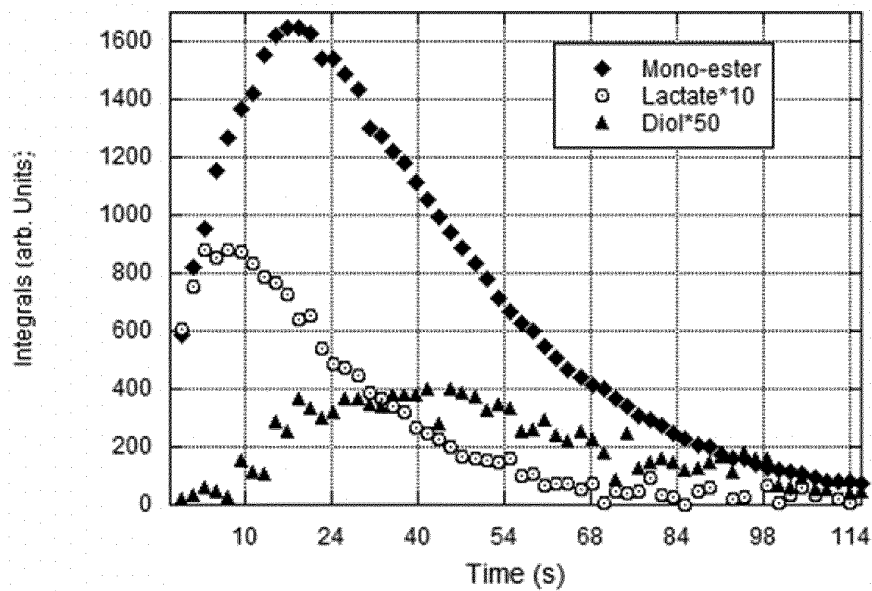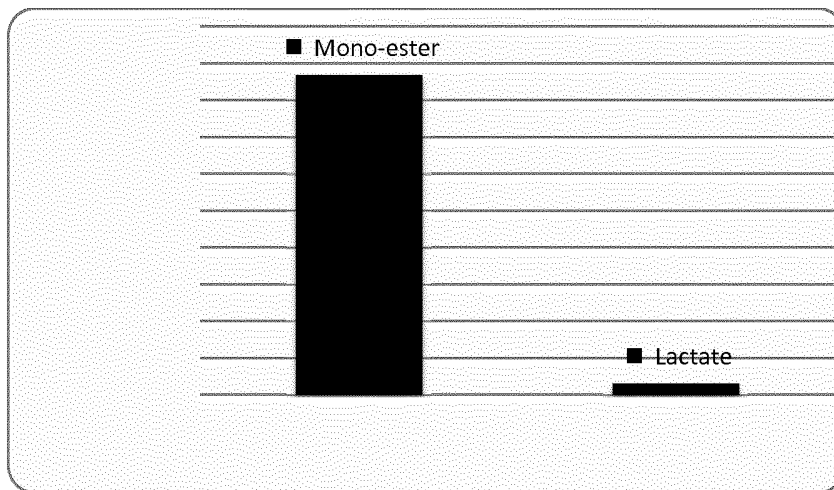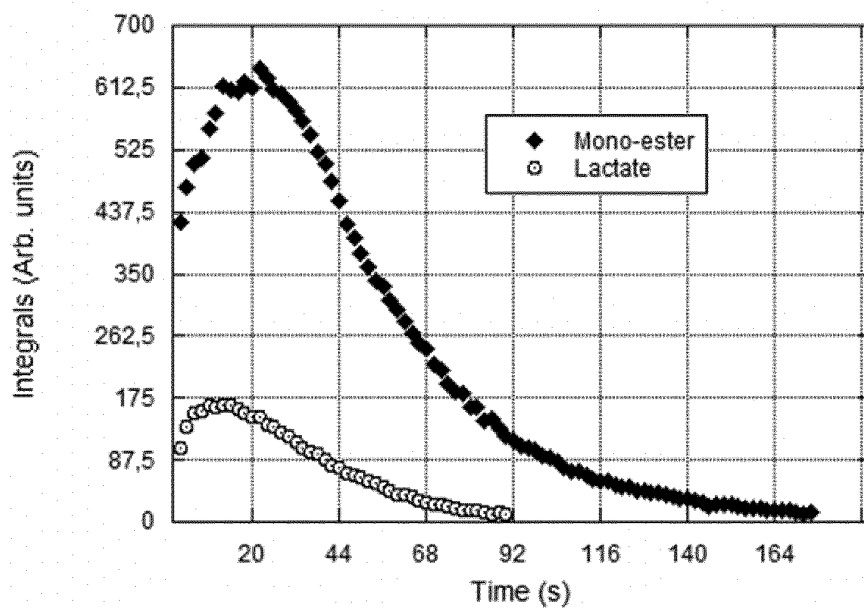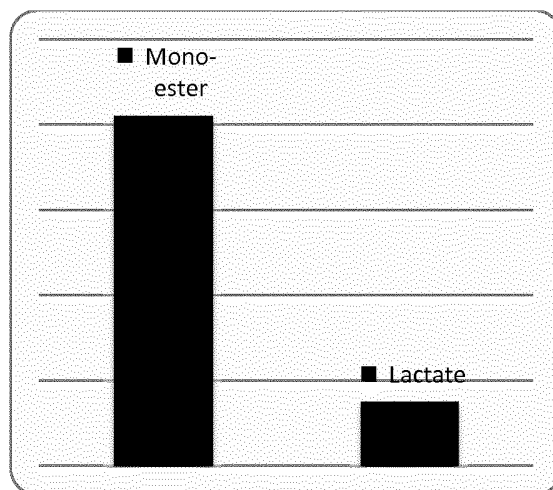b) removing the excess of acetyl chloride and the formed hydrochloric gas.
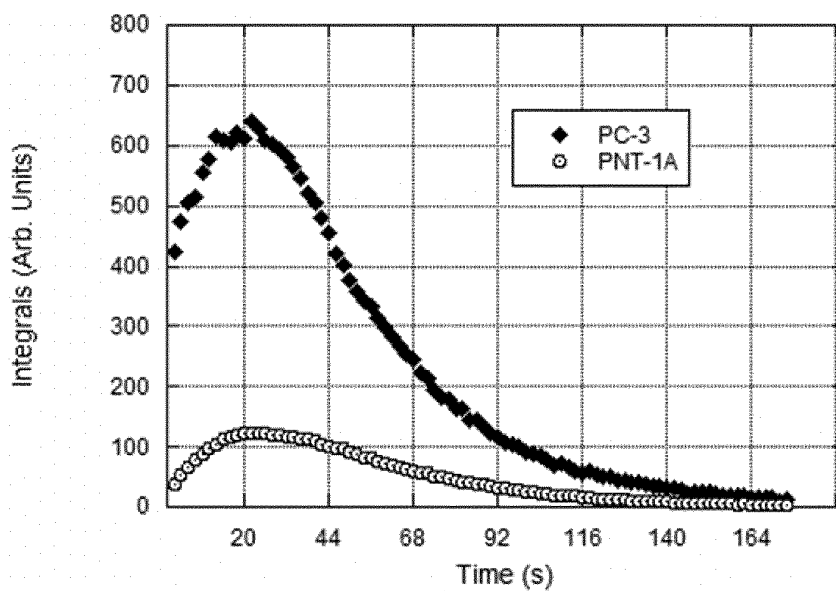
Figure 1

A

B

Figure 2

A
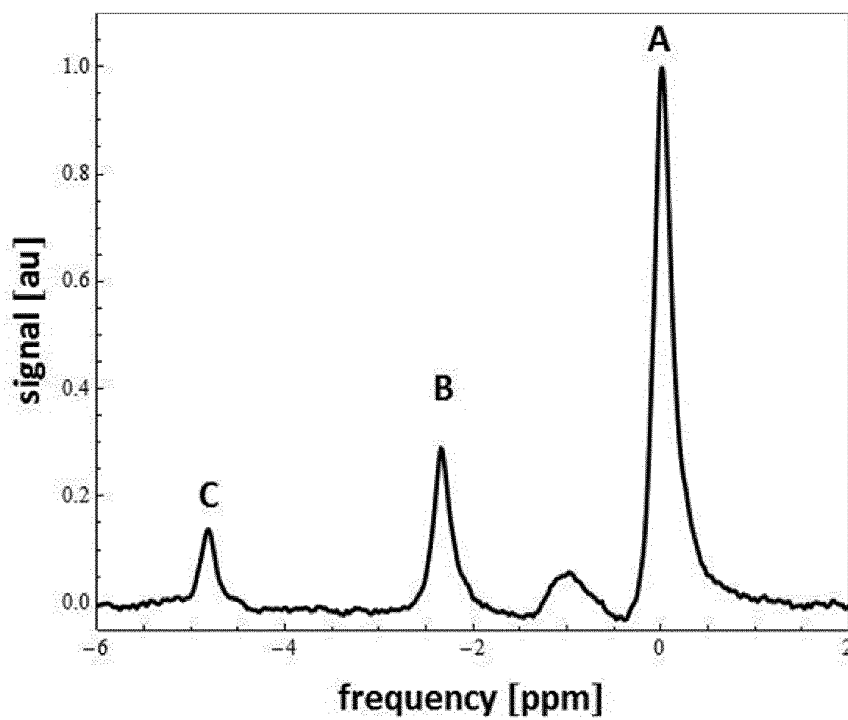

B

Figure 3

A
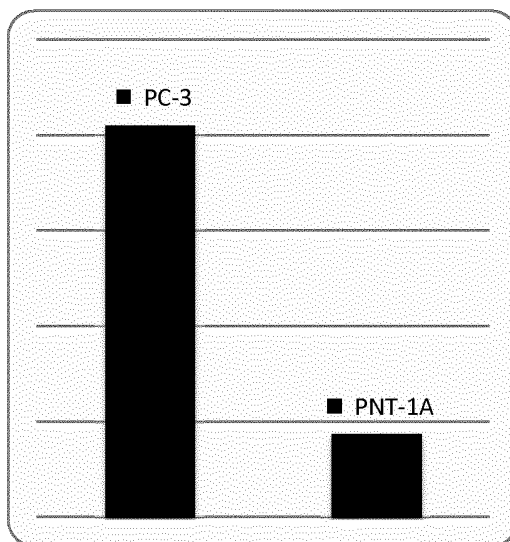
B

Figure 4
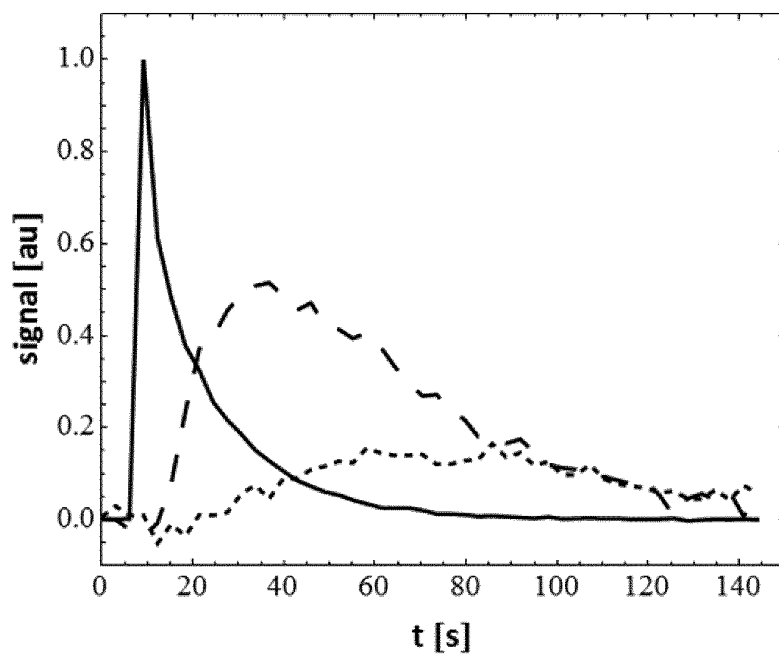
Figure 5

Figure 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K49/10
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)

A61K

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, BEILSTEIN Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2011/124672 A1 (BRACCO IMAGING SPA [IT]; AIME SILVIO [IT]; GIOVENZANA GIOVANNI BATTIST) 13 October 2011 (2011-10-13) cited in the application the whole document -----	1-18
A	US 7 271 268 B1 (SUEN RUNG-TIAN [TW] ET AL) 18 September 2007 (2007-09-18) example 2 -----	1-18
A	WO 99/35508 A1 (NYCOMED IMAGING AS [NO]; COCKBAIN JULIAN [GB]; ARDENKJAER LARSEN JAN H) 15 July 1999 (1999-07-15) cited in the application the whole document -----	1-18

| | Further documents are listed in the continuation of Box C. | [X] | See patent family annex. |

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2014	29/09/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bettio, Andrea

2

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 2011124672	A1	13-10-2011	CN	102892434 A	23-01-2013
			EP	2555803 A1	13-02-2013
			JP	2013523298 A	17-06-2013
			US	2013096420 A1	18-04-2013
			WO	2011124672 A1	13-10-2011
US 7271268	B1	18-09-2007	NONE		
WO 9935508	A1	15-07-1999	AU	752308 B2	12-09-2002
			AU	1775399 A	26-07-1999
			BR	9813244 A	10-10-2000
			CA	2317526 A1	15-07-1999
			CN	1285044 A	21-02-2001
			CN	1527066 A	08-09-2004
			EP	1046051 A1	25-10-2000
			EP	2119457 A1	18-11-2009
			ES	2393833 T3	28-12-2012
			HK	1069635 A1	29-02-2008
			HU	0102093 A2	28-09-2001
			JP	4764548 B2	07-09-2011
			JP	2002501006 A	15-01-2002
			NO	20003251 A	22-06-2000
			NZ	505151 A	26-11-2002
			RU	2221255 C2	10-01-2004
			US	6278893 B1	21-08-2001
			US	6466814 B1	15-10-2002
			WO	9935508 A1	15-07-1999