

PRODUCTION OF HIGHLY ISOTOPICALLY LABELLED SECONDARY
MICROBIAL METABOLIC PRODUCTS, AND CORRESPONDING METABOLIC
PRODUCTS

5 The present invention relates to a method for producing isotopically labelled secondary metabolic products of fungi or bacteria in a liquid synthetic culture medium as well as isotopically labelled secondary metabolic products of fungi or bacteria.

10 Today, isotopically labelled substances are of increasing importance, in particular in the technology of liquid chromatography with mass spectrometric detection (LCMS), which enables efficient spectrometric analyses at high

15 product throughputs. This technology can be used for a great variety of potential analytes without imposing any limitations as to the molecular mass, yet with possible problems occurring in the detection of the individual substances to the effect that both the disintegration

20 spectra and the individual molecule peaks have to be assigned accordingly. In order to ensure a reliable LCMS application and method, the use of so-called internal standard substances has become increasingly important. Internal standards are substances strongly resembling the

25 target analytes proper, i.e., in particular, possibly having identical molecular structures yet at different molecular weights. Isotopically labelled molecules of the target analyte, i.e. molecules in which one or several atoms in the molecule are replaced by their isotopes,

30 therefore, have turned out to be ideal internal standards. At present, such substances are produced by organic syntheses, for instance, by substituting hydrogens or carbons with the corresponding, heavier isotopes.

In this context, it has, however, been shown in LCMS
analyse that it is desirable that the isotopically labelled
substances used as internal standards have molecular mass
5 differences of at least 3 in order to enable the distinct
separation from the target analytes, and that, if possible,
substances comprising as few isotopomers as possible are to
be used.

10 A way of producing isotopically labelled plant or microbial
metabolites is via the biosynthetic path of the respective
plants and/or microbes. In doing so, culture media are
supplemented with radioactively labelled nutrients and the
culture medium components are to a certain percentage
15 integrated in the anabolic and metabolic cycles of the
microbial or plant cultures such that isotopes will be
incorporated in the metabolic products. That method
involves the drawback that only incomplete labelling is
feasible by this method and that a mixture of different
20 isotopomers is normally formed, what makes such
isotopically labelled substances, or isotopically labelled
plant or microbial metabolites, hardly suitable for use as
internal standards, since with the use of such substances
not one standard but a broad spectrum of isotopomers would
25 be applied, which would, in turn, render the selective
detection of target substances in LCMS spectrometries
possible not at all or only with great difficulty.

The present invention aims to provide a method for
30 producing isotopically labelled secondary metabolic
products of fungi or bacteria, in which all or almost all
of the carbon atoms, nitrogen atoms or sulphur atoms
contained the starting product are replaced by stable

isotopes, thus providing a single, isotopically labelled end product to be readily and reliably detectable in spectrometric processes, in particular LCMS. The invention further aims to produce a metabolic product which can be
5 safely and reliably used as an internal standard in spectrometric analytical processes, in particular LCMS.

To solve these objects, the method according to the present invention is conducted in a manner that the synthesis is
10 carried out by immobilizing the fungi or bacteria on an inert carrier while adding a liquid synthetic culture medium in which substantially all of the carbon atoms, nitrogen atoms and/or sulphur atoms have been replaced by stable isotopes. By realizing the synthesis by immobilizing
15 the fungi or bacteria on an inert carrier while adding a liquid synthetic culture medium in which substantially all of the carbon atoms, nitrogen atoms and/or sulphur atoms have been replaced by stable isotopes, it has become feasible to produce an isotopically labelled metabolic
20 product of the fungi and bacteria, in which all of the atoms, or at least 95% of the atoms, to be obtained from the culture medium by growing, i.e. carbon, nitrogen or sulphur atoms, have been replaced by the more stable isotopes of the isotopically labelled nutrients contained
25 in the culture medium so as to allow for the recovery of a selective, isotopically labelled product rather than a mixture of different homologs having varying numbers of isotope atoms, as was frequently described in the prior art. It is, thus, feasible by this production method to
30 obtain an isotopically labelled secondary metabolic product which can be selectively used and which can be clearly and unambiguously detected in any analysis, even in metabolic studies.

According to a further development, the method is conducted in a manner that sugars or sugar alcohols, in particular D-[U- $^{13}\text{C}_6$]-glucose, ^{13}C -sucrose, ^{13}C -glycerol and/or ^{13}C -acetate
5 are used as carbon sources in the liquid synthetic culture medium, ^{15}N -amino acids, -nitrates, -ammonium compounds or -urea are used as nitrogen sources, ^{33}S - or ^{34}S -sulphates, -sulphides or -amino acids are used as sulphur sources. When growing metabolic products of fungi or bacteria, the
10 fungus or the bacterium, respectively, due to completely labelled carbon, nitrogen and/or sulphur sources being contained in the liquid synthetic culture medium, will be forced to incorporate into the metabolic product the respectively labelled isotope so as to ensure that the
15 secondary metabolic products of the fungi or bacteria will be labelled with the respective isotopes, or replaced by the respected isotopes, to a high degree, if not completely.

20 In order to improve the yield of isotopically labelled secondary metabolic products of fungi or bacteria, the method according to a further development is conducted in a manner that the liquid synthetic culture medium additionally contains a mixture selected from inorganic
25 salts or acids and bases having the ions Na^+ , K^+ , Ca^{++} , Mg^{++} , Fe^{+++} , Zn^{++} , Cu^{++} , B^{+++} as well as CO_3^{--} , SO_4^{--} , PO_4^{---} , NO_3^- . Due to the fact that salts or acids and bases having the ions Na^+ , K^+ , Ca^{++} , Mg^{++} , Fe^{+++} , Zn^{++} , Cu^{++} , B^{+++} as well as CO_3^{--} , SO_4^{--} , PO_4^{---} , NO_3^- are contained in the liquid synthetic
30 culture medium, it is ensured that any foreign ions possibly present in the fungi or bacteria in addition to carbon, hydrogen, nitrogen and sulphur will be safely

provided in sufficient quantities so as to ensure high yields besides rapid growth.

In order to further improve the yield, a natural or
5 synthetic carrier having a large internal surface area, in particular silicate, layered silicate, zeolite, bentonite, burnt clay, diatomaceous earth, synthetics or the like, is used as said inert carrier. By using an inert carrier having a large internal surface area, it is feasible to
10 improve the yield in the method according to the invention by at least 50% as opposed to conventional methods, which are carried out without inert carriers having large internal surfaces areas. Such increases in yield not only render the method more economical, but also ensure that
15 sufficient quantities of the desired end products of the isotopically labelled secondary metabolic products will be produced so as to enable the same to be used in a suitable manner as internal standards in analyses, or even in metabolic studies.

20

According to the invention, the greatest improvements in yield will, in particular, be obtained in that an aluminium silicate, e.g. diatomaceous earth, in particular
kieselguhr, isolate HM-N or a zeolite, or a layered
25 silicate, in particular a vermiculite, from the group of mica minerals is used in natural or treated form as said inert carrier. With these substances, the surface properties such as surface tension, porosity and the like are, in particular, suitable to achieve especially good
30 turnover rates on the carrier surfaces. In an analogous manner, the use of inert synthetic carriers, which may be selected from foamed materials, polyamide, silicone, polyethylene, polypropylene, polytetrafluoroethylene, polyester

or the like, will allow for accordingly large improvements in yield, whereby the use of natural carriers having large internal surface areas, or the use of synthetic carriers, will produce similarly enhanced yields as a function of the
5 metabolic products to be produced.

For as rapid a method control as possible at a simultaneously high yield, the invention is further developed to the extent that the production is realized at
10 temperatures ranging between 3 and 45°C, in particular between 10 and 35°C. In this context, it has partially turned out to be favourable that a production method is not always conducted at a constant temperature, but that temperature variations within the indicated limits may also
15 lead to improved yields or accelerated reaction rates or elevated turnover numbers.

In order to obtain an end product as pure as possible, the method according to the invention is conducted in a manner
20 that the isotopically labelled secondary metabolic products are recovered from the liquid synthetic culture medium by extraction and concentration, for instance by a combination of steps like solid/liquid-liquid/liquid extraction, centrifugation, filtration and evaporation. After the
25 recovery of the isotopically labelled secondary metabolic products, it has turned out to be advantageous to subject these products to a further purification procedure, wherein, according to the invention, chromatographic methods and, in particular, column chromatography,
30 preparative thin-layer chromatography, ion chromatography, affinity chromatography, exclusion chromatography and/or preparative high-pressure liquid chromatography are preferably used as purification procedures. Such a

reprocessing method and purifying procedure will render feasible the recovery of isotopically labelled secondary metabolic products of fungi and bacteria, in which at least 95% of the carbon atoms, nitrogen atoms and/or sulphur atoms have been replaced with the respective stable isotopes, thus enabling the recovery of products with appropriate mass differences relative to their natural analytes so as to be sufficiently distinguishable from naturally occurring, heavy isotopes, for instance in a liquid chromatography with mass-spectrometric detection (LCMS), and, hence, for instance, allow for the provision of stable, clearly identifiable internal standards in such analyses.

The invention further aims to provide an isotopically labelled secondary metabolic product of fungi and bacteria, which comprises substantially all, in particular at least 95%, of the carbon atoms, nitrogen atoms and/or sulphur atoms replaced by stable isotopes.

20

According to a further development of the invention, such isotopically labelled secondary metabolic products of fungi or bacteria can be used as internal standards in analytics, for metabolic studies in animal feeding tests, for metabolic studies, for elucidating metabolic cycles, degradation paths and/or degradation periods as well as intercalations. For all of the mentioned purposes of use, it is of essential importance to have obtained a stable and clearly detectable standard, or a clearly detectable and trackable substance, in the test scheme or degradation scheme in order to be able to precisely reproduce the individual method or processing steps.

According to a further development, mycotoxins, in particular trichothecenes such as nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl deoxynivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, DAS, 5 fumonisins such as fumonisin B1, B2 or B3, ochratoxins such as ochratoxin A, B, C or D, zearalenones, moniliformin or aflatoxins such as aflatoxin B1, B2, G1 or G2 are used as metabolic products in analytical methods or in metabolic studies, degradation paths and the like. Mycotoxins are of 10 increasing importance in the etiology of animal diseases, and it is necessary to technically produce sufficient quantities of such substances in order to be able to subsequently carry out the respective toxicological veterinary examinations by using chemical substances as 15 distinct and pure as possible. Since mycotoxins constitute serious health risks to men and animals, their analytics is a theme of global interest, since, in particular, many countries have already developed guide and limit values for the tolerance of such substances. The detection and 20 quantification of such mycotoxins via the use of internal standards that are precisely detectable and, hence, enable the quantitative analysis of the respective toxin constitute an important advance in the detection of such noxious substances.

25

Similarly, also the quantitative detection or tracking of toxins and, above all, as in correspondence with a further development of the invention, endoxins and exotoxins, in particular bacterial toxins of *Escherichia coli* sp., *Sal-* 30 *monella* sp., *Clostridium* sp., *Bacillus* sp. or *Staphylococcus* sp., is of vital interest for the public health and for the detection of harmful substances in food and semi-luxury food. Also the use of metabolic products

such as antibiotics and, in particular, antibiotics formed of actinomycetes, like tetracyclines, streptomycines or aminoglycosides, antibiotics formed of *Bazillus* sp., like bacitracin or polymyxin, antibiotics formed of penicillium, 5 like penicillin or griseofulvin, or cephalosporins formed of cephalosporium, is increasing in importance, in particular in the event of diseases or for the detection of such substances in food and semi-luxury food, wherein it also holds for these substances that substances rendering 10 feasible the quantitative detection of metabolic products like antibiotics are of vial interest for the general public.

According to a further development, pure substances having 15 labelling degrees of ^{13}C , ^{15}N , or ^{33}S or ^{34}S are used as metabolic products, thus, on the one hand, enabling the clear differentiation from unlabelled substances or metabolic products and, on the other hand, also safely ensuring the differentiation from naturally occurring 20 isotopes and, finally, providing a substance to be precisely trackable in analyses and detection processes.

In the following, the invention will be explained in more detail by way of examples illustrating the production of 25 highly isotopically labelled metabolic products.

Example 1

Production of highly isotopically labelled $[\text{U-}^{13}\text{C}_{15}]$ -deoxynivalenol (DON)

30

To produce completely labelled ^{13}C -DON, a fusarium fungus, namely *Fusarium graminearum*, is inoculated on an inert carrier material, namely vermiculite, and incubated in a

synthetic culture medium consisting of 0.5 g K_2HPO_4 , 2.0 g $NaNO_3$, 0.7 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl , 15 g D-[U- $^{13}C_6$]glucose, 1.5 g $NH_4H_2PO_4$, 15 mg $Fe(II)SO_4 \cdot 7H_2O$ or 20 mg $ZnSO_4 \cdot 7H_2O$ and containing D-[U- $^{13}C_6$]glucose as the sole carbon source.

5 After 5 weeks at about 28°C, the toxin-containing material is extracted with ethyl acetate and subsequently purified to standard quality (purity >98%) by means of extraction, chromatography and crystallization.

10 About 40 ml incubation medium is prepared per production formulation. After this, pooled toxin-containing material is further processed. From 1000 ml formulation or batch, between 5 and 50 mg [7.5 to 17.5mg] completely labelled [U- $^{13}C_{15}$]-DON is obtained.

15

The purified product was characterized by the following analytical processes:

1H NMR and ^{13}C -NMR

LC-MS/MS Q Trap for determining the ^{13}C isotope portion

20 Determination of purity and concentration against reference materials using UV/VIS and HPLC-DAD

Adjustment of concentration

Quality control using UV/VIS, HPLC-DAD, LC-MS/MS A Trap

25 Such a highly isotopically labelled $^{13}C_{15}$ -deoxynivalenol ($^{13}C_{15}$ -DON) may, for instance, be used as an internal standard. Such an internal standard has a molecular mass that is heavier by exactly 15 g/mol, its signal in the mass spectrum (Fig. 1), thus, appearing exactly 15 amu higher
30 than the signal of the analyte. Since all of the other chemical and physical properties of the ^{13}C -labelled DON are identical with those of the analyte, such an internal standard shows exactly the same fragmentation as the

analyte, also the ionization of the substance is identical and, hence, even the ionization yield. This means that the signal heights of the fragments or substance ions become absolutely comparable between the internal standard and the
5 analyte, and, because the concentration of the internal standard is known in an analysis, direct conclusions can be drawn as to the concentration of an analyte, which is why such a highly labelled deoxynivalenol constitutes a near-ideal internal standard.

10

Fig. 1 shows C_{12} -DON and C_{13} -DON in a collective mass spectrum. In addition to the molecular peaks of $^{13}C_{15}$ -DON and ^{12}C -DON at 295.2 and 310.2, respectively, Fig. 1 also indicates the distribution of the compounds in which not
15 all of the C-atoms have been labelled and, hence, do not consist of one isotope species (isotopomers). In the case of naturally occurring deoxynivalenol, this is $^{13}C_1$ -DON, which corresponds to the natural distribution between C_{12} and C_{13} .

20

Example 2

Production of highly isotopically labelled ^{13}C -fumonisin

To produce a fumonisin completely labelled with ^{13}C , 1000
25 ml of a liquid medium consisting of 0.5 g KH_2PO_4 , 0.5 g KNO_3 , 0.7 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl , 17.5 g D-[U- $^{13}C_6$]glucose, 1.5 g $NH_4H_2PO_4$, 15 mg $Fe(II)SO_4 \cdot 7H_2O$ and 20 mg $ZnSO_4 \cdot 7H_2O$, with D-[U- $^{13}C_6$]glucose as the sole carbon source, applied on 1 x 1 x 1 cm foam cubes, are inoculated with *Fusarium*
30 *moniliforme* and incubated in an incubator at 28°C and 70% relative humidity. After 3 weeks, the toxin-containing material is extracted with a solvent mixture containing 1:1 acetonitril: H_2O and subsequently purified to standard

quality (purity >98%) by means of extraction and chromatographic steps such as ion exchange chromatography, flash column chromatography with silica gel, thin-layer chromatography and preparative HPLC.

5

In this manner, 80-240 mg ^{13}C -fumonisins are obtained per 1000 ml formulation (HPLC-FLD).

Example 3

10 Production of highly isotopically labelled [U- $^{13}\text{C}_{17}$]-3-acetyl deoxynivalenol

To produce highly isotopically labelled [U- $^{13}\text{C}_{17}$]-3-acetyl deoxynivalenol, 1000 ml of a synthetic liquid medium
15 consisting of 0.5 g KH_2PO_4 , 0.5 g KNO_3 , 0.7 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g KCl , 17.5 g D-[U- $^{13}\text{C}_6$]glucose, 1.5 g $\text{NH}_4\text{H}_2\text{PO}_4$, 15 mg $\text{Fe(II)SO}_4 \cdot 7\text{H}_2\text{O}$ and 20 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and containing completely isotopically labelled [U- $^{13}\text{C}_6$]-glucose as the sole carbon source, applied on diatomaceous earth, namely
20 isolate HM-N, are inoculated with *Fusarium graminearum* and incubated in an incubator at 28°C for 9 days. After 9 days, the toxin-containing material is harvested, extracted with acetonitril/ H_2O azeotrope and subsequently purified to standard quality (purity >98%) by means of extraction,
25 chromatography, crystallization, Büchi-MPLC and recrystallization. From one batch, about 15-50 mg of a highly pure end product can be obtained. The purity check is preformed by LC-UV analysis using a C18-capillary column.

30

An isotopically labelled 3-acetyl deoxynivalenol produced in this manner has a molecular mass that is heavier by 17 g/mol than that of unlabelled or not thoroughly or

incompletely labelled 3-acetyl deoxynivalenol. Incompletely
labelled 3-acetyl deoxynivalenol has a molecular mass of
M/z = 338, whereas the completely isotopically labelled
product has a molecular mass of 355. Fig. 2 shows the mass
5 spectrum of pure ^{13}C -3-acetyl-deoxynivalenol, from which it
can be seen that the product has been labelled by 75% and
the isotope distribution of the product is apparent. The
distribution of the product and the incompletely labelled
isotopomers in this case depends on the isotopic purity of
10 the starting product, $^{13}\text{C}_6$ -glucose, and can still be clearly
shifted towards a completely labelled product when using
completely pure $^{13}\text{C}_6$ -glucose. From Fig. 2 it is, however,
clearly apparent that isotopomers having less than 13 ^{13}C -
atoms are virtually absent such that ^{13}C -3-acetyl-
15 deoxynivalenol can also be perfectly used as an internal
standard.

Example 4

Production of highly isotopically labelled [U- $^{13}\text{C}_{17}$]-15-acetyl deoxynivalenol

To produce highly isotopically labelled [U- $^{13}\text{C}_{17}$]-15-acetyl
deoxynivalenol, a culture medium consisting of 0.5 g
 K_2HPO_4 , 2.0 g NaNO_3 , 0.7 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g KCl , 15 g D-[U-
25 $^{13}\text{C}_6$]glucose, 1.5 g $\text{NH}_4\text{H}_2\text{PO}_4$, 15 mg $\text{Fe(II)SO}_4 \cdot 7\text{H}_2\text{O}$ and 20 mg
 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ is inoculated with *Fusarium graminearum* on a
coarse-grained phyllosilicate carrier and incubated at 28°C
in an incubator. After 9 days, the toxin-containing
material is harvested, extracted with ethyl acetate and
30 subsequently purified to standard quality (purity >98%) by
means of extraction, chromatography and crystallization.
Alternatively to crystallization, a further purification
step using preparative HPLC may also be applied.

About 30-60 mg highly pure target product can be obtained from one fermentation batch.

5 Example 5

Production of highly isotopically labelled [U-¹³C₁₅]-nivalenol or [U-¹³C₁₇]-fusarenon-X

To produce highly isotopically labelled [U-¹³C₁₅]-nivalenol
10 or [U-¹³C₁₇]-fusarenon-X, a liquid medium consisting of 0.5 g K₂HPO₄, 2.0 g NaNO₃, 0.7 g MgSO₄*7H₂O, 2.0 g KCl, 15 g D-[U-¹³C₆]glucose, 1.5 g NH₄H₂PO₄, 15 mg Fe(II)SO₄*7H₂O or 20 mg ZnSO₄*7H₂O with [U-¹³C₆]-glucose as the sole carbon source and inert phyllosilicate is inoculated with Fusarium
15 nivale and incubated at 28°C for 5 weeks. After this, the toxin-containing material is extracted with methanol and methylene chloride and subsequently purified to standard quality (purity >98%) by means of extraction, chromatography and crystallization. Alternatively to
20 crystallization, a further purification step using preparative HPLC may also be applied.

Example 6

Production of highly isotopically labelled [U-¹³C₂₀]-ochratoxin A
25 ratotoxin A

To obtain the target substance, the fungus Petromyces albertensis on an inert phyllosilicate carrier is fermented with a synthetic liquid medium consisting of 0.5 g K₂HPO₄,
30 2.0 g NaNO₃, 0.7 g MgSO₄*7H₂O, 2.0 g KCl, 15 g D-[U-¹³C₆]glucose, 1.5 g NH₄H₂PO₄, 15 mg Fe(II)SO₄*7H₂O or 20 mg ZnSO₄*7H₂O, which contains completely ¹³C-labelled glucose as the sole carbon source. The flasks are then incubated in

an incubator for 6 weeks at 28°C and 70% air moisture and subsequently extracted with toluene. The target substance is purified by column-chromatography and recrystallized as in the preceding Examples.

5

Example 7

Production of highly isotopically labelled [U-¹³C₁₈]-zearalenon

10 To produce highly isotopically labelled [U-¹³C₁₈]-zearalenon, 1000 ml of a liquid medium consisting of 0.5 g KH₂PO₄, 0.5 g KNO₃, 0.7 g MgSO₄*7H₂O, 2.0 g KCl, 17.5 g D-[U-¹³C₆]glucose, 1.5 g NH₄H₂PO₄, 15 mg Fe(II)SO₄*7H₂O and 20 mg ZnSO₄*7H₂O, with D-[U-¹³C₆]glucose as the sole carbon
15 source, applied on porous burnt clay in granular form, namely Seramis or Lecca, is inoculated with Fusarium semitectrum and incubated in an incubator at 28°C and 70% relative humidity. After 3 weeks, the toxin-containing material is extracted with pure petroleum ether and a
20 petroleum ether/ethyl acetate mixture of 4:1 and 2:1 and subsequently purified to standard quality (purity >98%) by means of extraction and chromatographic steps such as ion exchange chromatography, flash column chromatography with silica gel, thin-layer chromatography and preparative HPLC.

25

Example 8

Production of highly isotopically labelled ¹⁵N₅-rocquefortine C

30 To produce a rocquefortin C completely labelled with the nitrogen isotope ¹⁵N, 1000 ml of a liquid medium consisting of 0.8 g KH₂PO₄, 0.7 g MgSO₄*7H₂O, 1.0 g KCl, 17.5 g D-Glucose, 1.0 g ¹⁵NH₄¹⁵NO₃, 1.5 g NaH₂PO₄, 15 mg

Fe(II)SO₄*7H₂O, 20 mg ZnSO₄*7H₂O, with ¹⁵NH₄¹⁵NO₃ as the sole nitrogen source, applied on coarse kieselguhr, are inoculated with Penicillium commune and incubated in an incubator at 12°C and 70% air moisture. After 48 days, the toxin-containing material is extracted with an organic solvent consisting of 9:1 chloroform:methanol and subsequently purified to standard quality (purity >98%) by means of liquid-liquid extraction, flash column chromatography with silica gel, and preparative HPLC. Per 1000 ml formulation, 300 mg ¹⁵N₅-rocquefortin C is obtained (HPLC-FLD).

Example 9

Production of highly isotopically labelled ¹⁵N₂-³³S-penicillin

To produce a penicillin completely labelled with the nitrogen isotope ¹⁵N and the sulphur isotope ³³S, 1000 ml of a liquid medium consisting of 1.0 g K₂HPO₄, 0.2 g MgCl₂, 20.0 g D-glucose, 1.0 g ¹⁵NH₄¹⁵NO₃, 0.5 g Na₂³³SO₄, 1.5 g Na₂HPO₄, 5 mg Fe(II)Cl₂, 5 mg ZnCl₂, with ¹⁵NH₄¹⁵NO₃ as the sole nitrogen source and Na₂³³SO₄ as the sole sulphur source, applied on small foam cubes, are inoculated with Penicillium notatum and incubated in an incubator at 28°C and 70% air moisture. After 30 days, the toxin-containing material is extracted with ethyl acetate and subsequently purified to standard quality (purity >98%) by means of liquid-liquid extraction, flash column chromatography with silica gel, and preparative HPLC. Per 1000 ml formulation, 500 mg ¹⁵N₂-³³S-penicillin is obtained (HPLC-FLD).

CLAIMS

1. A method for producing isotopically labelled secondary metabolic products of fungi or bacteria for the production of an internal standard for analytical methods, for metabolic studies in animal feeding tests, for metabolic studies, for clarifying metabolic cycles, for studies of degradation paths or degradation periods or for studies of intercalations in a liquid synthetic culture medium, characterized in that the synthesis is carried out by immobilizing the fungi or bacteria on an inert carrier, while adding a liquid synthetic culture medium in which at least 95% of the carbon atoms, nitrogen atoms and/or sulphur atoms have been replaced by stable isotopes, namely ^{13}C , ^{15}N , or ^{33}S or ^{34}S .
2. The method according to claim 1, characterized in that sugars or sugar alcohols are used as carbon sources in the liquid synthetic culture medium.
3. The method according to claim 2, characterized in that the sugar or sugar alcohols are selected from the group comprising D-[U- $^{13}\text{C}_6$]-glucose, ^{13}C -sucrose, ^{13}C -glycerol and ^{13}C -acetate.
4. The method according to any one of claims 1 to 3, characterized in that ^{15}N -amino acids, nitrates, ammonium compounds or urea are used as nitrogen sources in the liquid synthetic culture medium.
5. The method according to any one of claims 1 to 4, characterized in that ^{33}S - or ^{34}S -sulphates, sulphides or amino acids are used as sulphur sources in the liquid synthetic culture medium.
6. The method according to any one of claims 1 to 5, characterized in that the liquid synthetic culture medium additionally contains a mixture selected from inorganic salts or acids and bases having the ions Na^+ , K^+ , Ca^{++} , Mg^{++} , Fe^{+++} , Zn^{++} , Cu^{++} , B^{+++} as well as CO_3^{--} , SO_4^{--} , PO_4^{--} and NO_3^- .
7. The method according to any one of claims 1 to 6, characterized in that the inert carrier comprises a natural or synthetic carrier having a large internal surface area.

8. The method according to claim 7, characterized in that the natural or synthetic carrier is selected from the group comprising silicate, layered silicate, zeolite, bentonite, burnt clay, diatomaceous earth and synthetics.
9. The method according to claim 8, characterized in that an aluminium silicate is used in natural or treated form as said inert carrier.
10. The method according to claim 9, characterized in that the aluminium silicate is selected from the group comprising a zeolite, a layered silicate and a vermiculite.
11. The method according to claim 7, characterized in that a foamed material is used as the synthetic carrier.
12. The method according to claim 11, characterized in that the foamed material is selected from the group comprising polyamide, silicone, polyethylene, polypropylene, polytetrafluoroethylene and polyester.
13. The method according to any one of claims 1 to 12 characterized in that the production is realized at temperatures in a range of from about 3°C to about 45°C.
14. The method according to claim 13, characterized in that the production is realized at temperatures in a range of from about 10°C to about 35°C.
15. The method according to any one of claims 1 to 14 characterized in that the isotopically labelled secondary metabolic products are recovered from the liquid synthetic culture medium by extraction and concentration.
16. The method according to claim 15, characterized in that the extraction and concentration are selected from a combination of steps including solid/liquid-liquid/liquid extraction, centrifugation, filtration and evaporation.
17. The method according to claim 16, characterized in that a chromatographic method is used as a purification process.
18. The method according to claim 17, characterized in that the chromatographic method is selected from the group comprising column chromatography, preparative thin-layer

chromatography, ion chromatography, affinity chromatography, exclusion chromatography, preparative high-pressure liquid chromatography or any combination thereof.

19. An isotopically labelled secondary metabolic product of fungi or bacteria produced by a method according to any one of claims 1 to 18.

20. The metabolic product according to claim 19, wherein said metabolic product comprises a mycotoxin.

21. The metabolic product according to claim 20 characterized in that said mycotoxin is selected from the group comprising trichothecenes including nivalenol, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl deoxynivalenol, fusarenon-X, T-2 toxin, HT-2 toxin, DAS, fumonisins including fumonisin B1, B2 or B3, ochratoxins including ochratoxin A, B, C or D, zeara-lenones, moniliformin and aflatoxins including aflatoxin B1, B2, G1 or G2.

22. The metabolic product according to claim 19, wherein said metabolic product comprises one or more toxins.

23. The metabolic product according to claim 22 characterized in that said toxins are endoxins and exotoxins selected from the group comprising bacterial toxins of *Escherichia coli* sp., *Salmonella* sp., *Clostridium* sp., *Bacillus* sp. or *Staphylococcus* sp.

24. The metabolic product according to claim 19, wherein said metabolic product comprises an antibiotic.

25. The metabolic product according to claim 24 characterized in that the antibiotic is selected from the group comprising antibiotics formed of actinomycetes, including tetracyclines, streptomycines or aminoglycosides, antibiotics formed of *Bacillus* sp., including bacitracin or polymyxin, antibiotics formed of penicillium, including penicillin or griseofulvin, and cephalosporins formed of cephalosporium.

26. A method for producing isotopically labelled secondary metabolic products of fungi or bacteria in a liquid culture medium substantially as herein described.

27. An isotopically labelled secondary metabolic product of fungi and bacteria substantially as herein described.