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Edited by

Ulrich Pleiss
Drug Metabolism and Isotope Chemistry, Bayer AG,
D-42096 Wuppertal, Germany

Rolf Voges
Preclinical Safety, DMPK-Isotope Section, Novartis Pharma AG,
CH-4002 Basel, Switzerland

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Centennial Lecture: Marie Curie

K.-H. Bremer and D. Gantz
Hoechst AG, HMR Preclinical Development
D-65926 Frankfurt/ Main

The last decade of the 19th century saw an extraordinary couple of closely related scientific discoveries and developments in physics and chemistry that influenced the course of history in a most remarkable way. Starting with Wilhelm Conrad Röntgens discovery of X-rays in 1895, followed 1896 by Henri Becquerels finding that uranium salts emit a new type of radiation and cumulating 1898/99 in the reports of Marie Curie that this radiation, called radioactivity, is a more general phenomenon to be found also in other compounds e.g. the 'new' elements like Radium and Polonium.

These findings and subsequent progress in the work on the new phenomena were not made by chance or serendipity alone but came as the fruits of extremely hard work and combination of scientific expertise with an unbending will to dig into the secrets of nature.

It is the story of the life of Maria Sklodowska-Curie that provides deep insights into the roots of radiochemistry and modern physics. And looking back about 100 years ago one is stunned as to what Marie Curie and her congenious husband Pierre were able to achieve under the most unfavorable conditions for scientific work.

At her time as well as today, Marie Curie is to be regarded as one of the outstanding women of science and is honoured now on the occasion of the 100th anniversary of her first success in the area of radiochemical work by a specially dedicated lecture about her life.

The Discovery of Polonium and Radium 1898 and the Consequences in Physics and Chemistry

W. Dedek, Leipzig

The pioneer work of Marie and Pierre Curie, starting with the observation of much greater activities in natural pitchblende rather than in chemical pure uranium compounds is reviewed between 1898 - 1902; the following topics, of later radium research also, will be discussed in detail:

- the mysterious observations on Po and the Bi-Te controverse between Marie Curie and W. Marckwald
- the preconcentration of Ra from Ba and the contributions of St. Meyer, E. Rutherford, and F.O. Giesel in early radium research
- a review of the discoveries on Ra including the controverse between Rutherford and Ramsay and Soddy, energy production, half-life, mother and daughter, the active deposit, atomic weight and radium standards, the historical concern of the Ra isotopes 224 (Th X) and 228 (MsTh I), and the industrial production of Ra
- the famous experiments of Ramsay and Soddy with Ra-emanation

A further review is given of great discoveries in physics and chemistry, derived from the studies using the alpha-radiation of polonium and radium, including

- the famous scattering experiments of Rutherford, Geiger, and Marsden and the detection of the atomic nucleus by using Ra-emanation, Po and short-lived Po isotopes
- the results of the alpha-irradiation of Be and Al and the discovery of the neutron in 1932 and artificial radioactivity in 1934; Po, Ra and Emanation as neutron-sources
- the atomic models of J.J. Thomson, E. Rutherford, and N. Bohr and the perception of isotopy
- the indicator method of G. Hevesy 1912
- the emanation method of O. Hahn 1926

Finally a brief review of radium toxicity and alchemistic "pharmaceuticals" containing radium will be given.

Parahydrogen and Orthodeuterium as Screening Tools to Optimize the Catalytic Exchange of Tritium in Drugs

J. Bargon

Institute of Physical Chemistry, University of Bonn, 53115 Bonn, Germany

The catalytic exchange of hydrogen against tritium in final drugs or precursors can significantly speed up their isotopic labeling for metabolic studies. Various catalysts have been found to mediate such a catalytic exchange, but the screening process to determine their efficiency and selectivity can be cumbersome as well as time-consuming, it requires a certified isotope laboratory, qualified personel, and it can cause radioactive waste.

A magnetic labeling technique instead using the para- or ortho-spin isomers of regular H_2 or D_2 in combination with in situ NMR spectroscopy allows to quickly screen and optimize the catalysts while yielding instant information about their selectivity and efficiency without envolving any radioactivity. Therefore, such studies can be carried out without an isotope facility.

APPLICATIONS OF NEW CATALYTIC PROCEDURES FOR THE DEUTERIATION AND TRITIATION OF ORGANIC COMPOUNDS

J. R. Jones and S.-Y. Lu

Chemistry Department, University of Surrey, Guildford GU2 5XH, UK

Although the use of deuterium and tritium labelled compounds in both the physical and life sciences continues to increase the methods that are used to prepare the compounds are well-established (Table 1) and many have been in operation for close on half a century. This is not to say they are ideal as some of them, particularly when tritium is employed, have serious limitations/disadvantages. This is compounded by the fact (Table 2) that there are pressures both within universities and industry, for chemical reactions to be performed in a cleaner, more efficient and environmentally friendlier manner.

Table 1 Widely Used Deuteriation/Tritiation Procedures

Method	Comments
1. Hydrogenation of an unsaturated precursor using either a homogeneous or heterogeneous catalyst and D_2 or T_2 gas	Both D_2 and T_2 are sparingly soluble in many common solvents so that the reactions are frequently slow. There are also problems in storing T_2.
2. Catalytic dehalogenation of aromatic halides using either D_2 or T_2 gas	Frequently only one deuterium or tritium atoms are incorporated. Similar problems encountered to hydrogenations.
3. Reduction of suitable precursors with labelled borohydrides	Tritiated borohydrides at high specific activity need to be prepared immediately prior to use.
4. Methylations using *e.g.* CD_3I, CT_3I	Range of compounds somewhat restricted. CT_3I not very stable and easy to use.
5. Hydrogen isotope exchange reactions—catalysed by acids, bases or metals	In the case of tritium specific activity somewhat limited as T_2O can not be readily used. Regiospecificity needs to be improved.

Table 2 Legislative/Commercial Issues

1. Tighter controls on how much radioactivity can be released to the atmosphere
2. Increasing cost associated with storage and dispensing of radioactive waste
3. The image of the chemical industry

The deuteriation/tritiation procedures most commonly used invariably require a catalyst and in view of the important developments that have been made in catalysis over the last 10-20 years

(Table 3) it seems sensible to investigate whether these can be used to benefit deuteriation/tritiation procedures.

Table 3 Important Developments in Catalysis Which Could Benefit Deuteriation/Tritiation Studies

1.	Phase Transfer Catalysis
2.	Supercritical Fluids
3.	Sol-Gel Technology
4.	Ionic-liquids
5.	Solid State Reactions
6.	Energy-Enhanced Reactions
7.	New Reagents

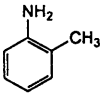
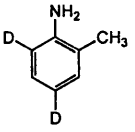
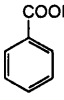
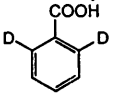
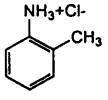
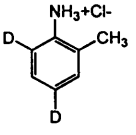
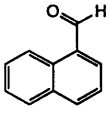
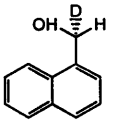
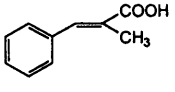
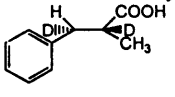
The present publication will concentrate on the benefits of using microwaves in a number of deuteriation/tritiation reactions (Table 4). The use of solid deuterium/tritium donors greatly increases the attractions of hydrogenation reactions. They can also be employed in the study of microwave enhanced dehalogenation reactions.

Table 4 Microwave–Enhanced Deuteriation/Tritiation Procedures

Reaction		References
1.	Hydrogenations using Solid Deuterium/Tritium Donors	1-3
2.	Catalytic Dehalogenations using Solid Deuterium/ Tritium Donors	4
3.	Solid State Catalytic Hydrogenations	5
4.	Solid State Sodium Borohydride/Borodeuteride Reductions	6
5.	Acid-Catalysed Deuteriation of Aromatic Amines	7,8
6.	Aromatic Decarboxylations	9

As the results in Table 5 make clear the saving in time for a variety of deuteriation, and by implication, tritiation reactions is considerable. Furthermore the use of microwaves opens up new possibilities *e.g.* reductions of substrates which hitherto are too slow or may not occur to any extent may now do so. Finally there is the possibility *via e.g.* decarboxylation reactions, to transfer labile deuterium or tritium into strategically more stable and useful positions. This could have considerable benefit in the treatment of *e.g.* radioactive waste.

Table 5 Examples of Rate Accelerations in Microwave-Enhanced Deuteriation Reactions

Reaction		Equilibrium/Reaction time	
Substrate	Product	Thermal Heating At ~100 °C	Microwave Conditions
1. Nafion NR50 Catalysed H/D Exchange		4 days	5 minutes
			
2. Polymer-supported $RhCl_3$ Catalysed H/D Exchange		24 hours	5 minutes
			
3. Homogeneous (HCl) Catalysed H/D Exchange		>24 hours	30 minutes
			
4. Borohydride ($NaBD_4$) Redcution		2 hours	2 minutes
			
5. Hydrogenation using Wilkinson's Catalyst and $DCOONH_4$		20 minutes	4 minutes
			

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High Specific Activity *n*-Tributyltin Tritide: A Versatile and Efficient Tritium Labelling Reagent

Manouchehr Saljoughian,* Hiromi Morimoto, Chit Than and Philip G. Williams, *The National Tritium Labelling Facility, and Physical Biosciences Division, Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley, CA 94720, USA.* Hendrik Andres, *Novartis Pharma AG, DMPK Isotope Section, CH-4002 Basel, Switzerland.*

Radical reactions are potentially useful as tritiation techniques because they are less subject to steric hindrance or rearrangement than ionic reactions. We have developed four different tritium labelling techniuques using high specific activity tributyltin tritide. A variety of organic compounds were selected and appropriate precursors were prepared. Radical-induced tritiation gave stereoselectively tritium labelled compounds with specific activities in the range of 625-810 GBq/mmole (17-22 Ci/mmol). The radiochemical purity of the products and specificity of the labelling techniques were examined with radio-HPLC, radio-GC and ^{1}H and ^{3}H NMR spectroscopy.

i. Radical-Induced Tritiodehalogenation: We recently developed N-tritioacetoxyphthalimide as a new high specific activity tritioacetylating reagent.[1] The tritiated reagent was synthesized from N-iodoacetoxyphthalimide at a specific activity of 480-630 GBq/mmole (13-17 Ci/mmole) using *n*-tributyltin tritide[2] and was characterized by ^{1}H and ^{3}H NMR spectroscopy, and by radio-HPLC. The efficacy of the product as a selective and facile tritioacetylating reagent was surveyed by labelling the free N-terminal amino group of several peptides. In comparison to tritiated acetic anhydride, N-tritioacetoxyphthalimide has several advantages. It is a nonvolatile solid, selectively delivering an acetyl group with the same specific activity as the reagent, and normally 1 to 1.5 equivalents affords rapid and complete acetylation under simple, mild basic conditions at 25°C. Reaction conditions were developed for the tritioacetylation of amino acids and peptides in a range of organic solvents (acetonitrile, dimethylsulfoxide, methanol, dioxane) and water.

ii. Radical-Induced Tritiodeoxygenation: High specific activity *n*-tributyltin tritide was used to effect selective tritium labelling through the free radical deoxygenation[3] of several biologically important compounds. Selective deoxygenation reactions require synthesis of precursors such as the 2'-*O*-phenoxythiocarbonate ester of adenosine and the 4-*p*-fluorophenoxy-thiocarbonate esters of cholesterol and *epi*-cholesterol. Once the appropriate ester derivative was prepared, the desired tritiated materials were readily produced in one step. Products were analyzed by radio-HPLC, a combination of GC/LSC for the chemical and radiochemical purity, and ^{1}H and ^{3}H NMR spectroscopy for the specificity of tritium labelling. In confirmation of deuterium labelling studies,[4] the synthesis of [2'-^{3}H]-2'-deoxyadenosine gave stereoselective incorporation of tritium [91% 2'-*R* (ribo), 9% 2'-*S* (arabino)], with a specific radioactivity of 750 GBq/mmole (20.2 Ci/mmole). Both the cholesterol and *epi*-cholesterol deoxygenation products had specific activities of *ca.* 810 GBq/mmole (22 Ci/mmole), and ^{3}H-NMR analysis showed the same ratio of [3-^{3}H] cholest-5-ene isomers (70% 3-α-axial, 30% 3-β-equatorial) independent of the stereochemistry of the starting ester. This is expected from a radical reaction mechanism, and our results confirm the previous deuterium studies with nucleosides.[4]

iii. Reductive cleavage of aliphatic nitro groups: Tertiary and activated secondary nitro compounds are readily denitrated by radical reduction with *n*-tributyltin hydride in the presence of azobisisobutyronitrile (AIBN) as initiator.[5] We have investigated the utility of this method in deuterium and tritium labelling chemistry by the reduction of several primary, secondary and tertiary α-nitroketones to the α-labelled ketones. Deuteriated products have been

analyzed with ^2H NMR, mass spectrometry and gas chromatography for the labelling specificity, deuterium content and chemical yields respectively. For the five deuteration reactions, chemical yields have ranged from 54-77%, and deuterium incorporation levels have been 43-65%. The analogous tritiation reactions using *n*-tributyltin tritide to tritiodenitrate the precursors are currently underway. Labelling using the radical denitration approach can be extended by use of Michael addition reactions.[5] One of our examples used Michael addition of acrolein to 2-nitrocyclododecan-1-one followed by radical denitration to give conjugate addition of a cyclic ketone, with specific labelling on the tertiary carbon. Similar examples are described in the literature.[5]

iv. Reductive cleavage of aliphatic amino groups: Primary, secondary, and tertiary aliphatic or alicyclic isocyanides, isothiocyanates and isoselenocyanates are smoothly and selectively reduced under free radical reaction conditions to the corresponding hydrocarbons using *n*-tributyltin hydride as a reducing agent.[6] The reaction is conceptually similar to the deoxygenation of primary and secondary alcohols (Barton reaction), and the selective replacement of a primary amino function by a hydrogen atom is a desirable reaction for the labelling of many natural products. We have applied this technique to the synthesis of 2-deoxyglucose and used glucosamine as the starting material. Glucosamine was liberated from the hydrochloride salt to free amine and then was N-formylated. The N-formylglucosamine was then peracetylated with acetic anhydride and the product was dehydrated to give 2-glucosamine isocyanide which was then isolated and purified for the labelling reaction. Cyanide abstraction at the 2-position with freshly made *n*-tributyltin deuteride in benzene at 80°C for 2 hours in the presence of a catalytic amount of AIBN generated tetra-*O*-acetyl-[2-^2H]-deoxyglucose which was analyzed with ^2H NMR spectroscopy to show 2 deuterium peaks at 1.5 and 2 ppm. Deacetylation in the final step generated [2-^2H]-deoxyglucose. This reaction will also lead to a general method for the synthesis of α-deuterium labelled carboxylic acids from amino acids as well. The analogous radical tritiodeamination reactions using *n*-tributyltin tritide are underway.

Conclusion: We have demonstrated our ability to produce high specific activity *n*-tributyltin tritide for the development of a variety of specialized and stereoselective tritium labelling methodologies. These approaches are important adjuncts to the traditional catalytic tritiation and tritiodehalogenation methods. *n*-Tributyltin tritide was applied to the synthesis of high specific activity N-tritioacetoxyphthalimide *via* a radical tritio-dehalogenation reaction and was also used for radical tritio-deoxygenation, tritio-denitration and tritio-deamination reactions to provide high specific activity tritiated molecules. These newly developed tritium labelling methodologies provide attractive and sophisticated approaches for selective tritium incorporation into molecules of biological importance. The full details of these exemplary radical-induced tritiation reactions will be published later.

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An Acidic Hydrocarbon as a Powerful Deuterium/Tritium Labeling Agent

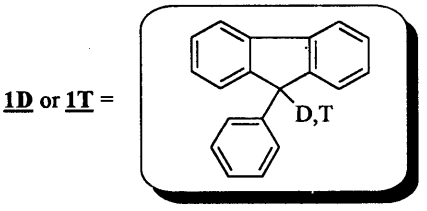
Jean-Christophe Cintrat and Bernard Rousseau
CEA/Saclay, DBCM/SMM bat. 547, 91191 Gif sur Yvette cedex (France)

One of the most convenient methodology for deuterium or tritium labeling is to run a classical isotope exchange reaction. Among the different methods available, one of the most popular is the acid-base pathway where an hydrogen atom is abstracted and replaced by one of its isotopes according to :

In such an approach no chemical modifications are required on the substrate but due to the difficulty to obtain and handle tritiated water at high specific activity this method is often precluded when elevated isotopic incorporation is needed. Moreover the same holds true for tritiated alcohols or carboxylic acids.

We have thus focused on a compound that should be easily accessible, stable, easy to handle and able to label efficiently a wide range of products.

To meet the above stated requirements, we have therefore chosen an acidic hydrocarbon (A-T) such as 1, *id est* 9-deutero (9-tritio), 9-phenyl fluorene which was our model substrate for this study :



1D 98.5 % i.e.
1T 24 Ci/mmol

A–D or A–T

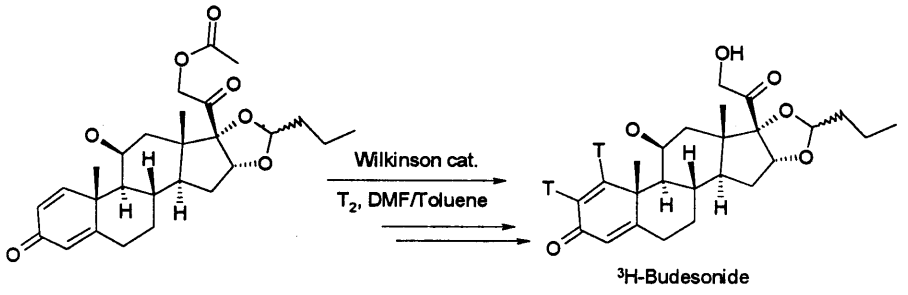
In this presentation, we will discuss our preliminary results in the field of acid/base reactions using 1 as a deuterium/tritium source. We will demonstrate how efficient, general and practical our methodology is since aliphatic, benzylic as well as aromatic positions can be specifically labeled with isotopic enrichment up to 91 %.

Synthesis of ^{3}H-Budesonide and multilabelled Cysteine

MARCO SKRINJAR* and PETER STRÖM
Department of Medicinal Chemistry, Preclinical R&D, Astra Draco AB
S-221 00 Lund, Sweden

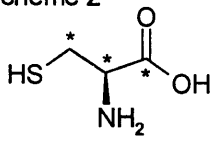
Budesonide, scheme 1, is a very potent anti-inflammatory acting corticosteroide which has an excellent effect in asthma treatment. It is well established on the market under the name Pulmicort® together with the inhalation device Turbuhaler®. The synthesis involves a selective double bond reduction which then is reintroduced. Even if the reintroduction involves the positions with tritium a high specific activity is achieved, 1.33 TBq/mmol.
The synthesis of the amino acid Cysteine, scheme 2, involves a number of synthetic steps that enables different positions to be labelled. The synthesis starts with ^{14}C- or ^{13}C-bromo-acetic acid and also involves an enzymatic resolution that results in a product with an e.e. > 99%.

Scheme 1



^{3}H-Budesonide

Scheme 2



* = ^{14}C or ^{13}C, ^{15}N

A CONVENIENT METHOD TO LABLE MAG-Phe-Leu-Gly-DRUG CONJUGATES

E. Fontana, A Pignatti, F.Orzi and F. Angelucci.

Pharmacia & Upjohn S.p.A., Drug Metabolism Research-Italy
Viale Pasteur 10, 20014 Nerviano (MI), Italy

HPMA-based polymers bearing a spacer covalently linked to cytotoxic agents have been designed to improve the antitumor efficacy, the solubility and the chemical stability of known cytotoxic drugs as well as to reduce their toxic side-effects [1].

In case of MAG-Phe-Leu-Gly-Camptothecin conjugates, the rationale is that the macromolecules are relatively selectively retained in the tumor compared to normal tissues. Once accumulated in the tumor mass, camptothecin (CPT) is released to its active form (lactone) by pH and enzyme mediated cleavage and excerts its effect preferentially in the tumor. In order to support this concept, the preparation of a radiolabelled form of these compounds was required to carry out whole body autoradiography studies in tumour bearing mice. The easy commercial availability of tritium labelled CPT seemed at first glance the quickest and most convenient approach to introduce the radiolabel into this class of compounds. The 3 step synthetic pathway involved the reaction of [^{3}H]CPT with a protected spacer followed by removal of the amino protective group and the linking of the radiolabelled side-chain to the MAG-ONP principal polymeric chain. However, previous stability studies carried out with commercially available tritium labelled CPT, showed a considerable and rapid loss of tritium both *in vitro* and *in vivo* [2]. Therefore, it was decided to perform a pilot tritium exchange study in the mouse with the same batch of [^{3}H]CPT that would have been used to prepare the tritiated MAG-Phe-Leu-Gly-Camptothecin conjugates. The results of the above study showed that the tritium exchange was low at least 3 days after administration to the animals. As a consequence, the radiolabelled material was considered suitable for WBA studies with tritium labelled CPT and/or CPT linked to a MAG carrier.

The same approach was successfully followed to prepare MAG-Phe-Leu-Gly-Taxol derivatives starting from the commercially available tritiated taxol.

Selected examples of MAG-Phe-Leu-Gly-Camptothecin and MAG-Phe-Leu-Gly-Taxol conjugates will be presented.

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Application of the Uranium-Tritium Manifold System in Labelling Chemistry

Peter Ström

Department of Medicinal Chemistry, Preclinical R&D, Astra Draco AB
S-221 00 Lund, Sweden

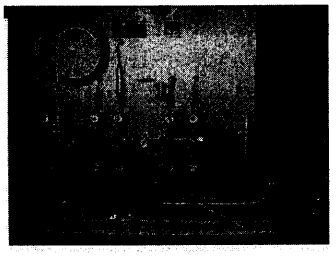


Fig.1 Uranium-Tritium Manifoldfrom RC-Tritec Switzerland. Designed by Peter Ström(Astra Draco), Albert Zeller(RC-Tritec) and Tom Werner(Astra Arcus)

- Pressure control, ≤ 4bar (precision 1mbar)
- Pure tritiumgas, no ^{3}He
- Easy handling and storage of T_2 on ^{238}U
- Easy to make test synthesis
- Reabsorbs unreacted tritiumgas
- Custom made, all parts replaceable
- Enable gas mixtures, e.g. T_2, D_2, H_2
- Enable tritide synthesis

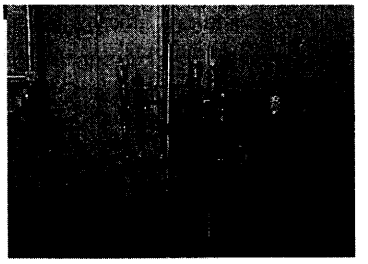


Fig. 2 Old Tritium-manifold (Töppler-pump)

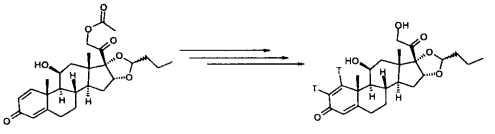
- No pressure control
- T_2-gas quality dependent of storage time
- No reabsorbtion of unreacted gas possible
- 3 kg of mercury involved
- Few replaceable parts
- Good for a few reactions a year
- Inexpensive and mobile

1 One example of the utility of the U-Tritium manifold is the reduction of Budesonide which could be achieved four times faster in comparison with the old Töppler-manifold. This is due to the ability to increase tritiumgas pressure. Furthermore, by changing from SeO_2 to the PhSeCl chemistry , the specific activity was increased to 1.33 TBq/mmol from 0.66 TBq/mmol.

2 The possibilty to make tritides by exchange reactions under controlled gas pressure and temerature, e.g $LiB^{3}H_4$ for reduction of carbonylgroups.

3 To be able to do reactions under controlled pressure to aviod side reations, as over-reduction of the product.

1



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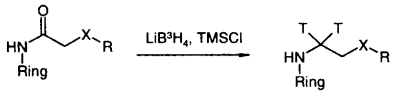

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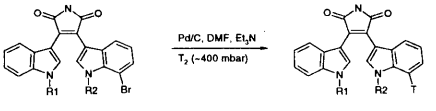

Security and Practical Aspects in Tritium Handling

K.Pfanner and A.Zeller
RC TRITEC AG, Speicherstrasse, 9053 Teufen, Switzerland.

Uranium beds are in common use for storage, transportation and purification of Tritium. Beds of various sizes and storage capacities as well as Tritium handling units are available from RC TRITEC AG. In Tritium labelling Uranium beds with small capacities and minimized volumes are used to store fresh Tritium and to liberate the Tritium required for a reaction. Uranium beds with large capacities are used to recover Tritium after use and flow-through Uranium beds are used in circulation systems for a more complete removal of traces of Tritium. Under normal operation conditions the Uranium beds may be operated over years. We would like to discuss what happens in the case of an air or solvent vapor ingress into the Uranium bed and give some handling principles to avoid accidents or damaging of the Uranium beds.
Pyrophoric Uranium readily reacts with Oxygen and solvents at room temperature and with Nitrogen at elevated temperatures according to[1,2]:

$$3\,U + 4\,O_2 \rightarrow U_3O_8 \qquad 1$$

$$4\,U + 3\,N_2 \rightarrow 2\,U_2N_3 \qquad 2$$

$$0.5\,U + 0.5\,UT_3 + 0.604\,N_2 + 0.145\,O_2 \rightarrow 0.291\,UO + 0.709\,UN + 0.5\,NH_3 \qquad 3$$

$$U + CH_4 \rightarrow UC + 2H_2 \qquad 4$$

All these Reactions are exothermic. In the case of an uncontrolled air ingress into a partially Tritium loaded Uranium bed one could expect a complete destruction of the bed and a liberation and partial combustion of the Tritium due to reactions according to the above equations and due to a resulting temperature rise. Experiments performed in several groups clearly show that this does not happen to that extend[1-5]. UT_3 does not react significantly with air. Air ingress into a partially loaded Uranium bed leads to a partial oxidation of the Uranium, but only traces of Tritium are set free. The temperature rise depends on the design of the bed but is not sufficient to liberate the bound Tritium. There are several reasons for the self limiting character of the reaction of Uranium with air: one is a blanketing effect by Tritium and/or inert gas (Argon, moisture) in air, another is the thermal conductivity and heat capacity of the bed, and a third reason is the formation of a diffusion barrier layer on the Uranium grains formed by the reaction products. Only in the special case of a continuous air flow through the pyrophoric Uranium powder very high temperatures above 1000°C occur and lead to a complete destruction of the bed and liberation of the Tritium. At such high temperatures the Uranium reacts with the steel and forms an alloy with an eutectic melting point of 725°C with easy to imagine consequences. Small amounts of air reduce the capacity of the bed (5.6 mmol O_2 destroy 1 g U) and the uptake speed for Tritium (especially at low pressures) is reduced due to passivation.
Any air ingress should therefore be avoided. Before expanding Tritium into the manifold system a leak test should be performed. Solvents should be degassed before Tritium is expanded into the reaction volume.
When pyrophoric Uranium is exposed to solvent vapors it immediately reacts according to equation 4. As a result part of the Uranium is destroyed, the capacity for Tritium uptake is

reduced, the unaffected part of the Uranium is partially passivated (this may in some cases lead to an almost complete blocking of further Tritium uptake) and the Hydrogen formed in the reaction dilutes the Tritium. The unreacted part of the Uranium may be reactivated in order to restore the full Tritium uptake speed, but the capacity of the bed remains reduced. Solvent vapors should therefore be avoided in order to preserve the full capacity of the Uranium bed and the purity of the Tritium.

Solvent vapors are usually removed by freezing the reaction mixture with liquid Nitrogen. Sometimes this procedure is not sufficient. During the reaction the Tritium gas is saturated with solvent vapor but only the solvent vapor in the reaction vessel is condensed. In order to remove all solvent the Tritium together with the solvent may be adsorbed onto Zeolite at liquid Nitrogen temperature. When the liquid Nitrogen cooling is removed the Tritium is desorbed whereas the solvent remains adsorbed. Pleiss et. al. use a similar procedure with activated carbon[6]. Polar solvents like Methanol are strongly adsorbed into Zeolites like for example Zeolite X even at room temperature. For non-polar solvents activated carbon or hydrophobic Zeolites are much better suited. We recommend a pre-dried mixture of Zeolite X and hydrophobic Zeolite which is activated in the Tritium manifold system prior to each experiment by evacuating at 250°C. Solvent vapors may be condensed into a cooled ampoule or just pumped off. The adsorption capacity of the adsorbents should be chosen such that the equilibrium pressure at liquid Nitrogen temperature is only a few mbar. From adsorption isotherms it can be concluded that for 100 Ci about 1 g of Zeolite mixture is required.

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A NEW STRATEGY OF DNA LABELLING WITH STABLE ISOTOPES

J.G. Baillon, N. Gilles, M. Courçon, A. Ménez and R. Genet

CEA Saclay, Département d'Ingénierie et d'Etudes des Protéines,
F-91191 Gif-sur-Yvette Cedex, France

In the past decade, multidimensional heteronuclear NMR has become essential to study the structure and dynamics of biological macromolecules and their complexes in solution. ^{2}H-, ^{13}C- and/or ^{15}N-enrichment of milligram quantities of recombinant proteins expressed in prokaryote or eukaryote cells already allowed to extend the scope of this technique up to *ca.* 35 kilodaltons molecules. However, beside protein labelling, there is a great deal of interest for oligonucleotide labelling in order to study their association with proteins whose role is crucial for the regulation of gene expression.

Here we propose a large scale and low cost methodology for the perdeuteration of DNA oligonucleotides, based on the polymerase chain reaction (PCR) technique. This strategy involves a three-step procedure: first *(i)*, the production of perdeuterated biomass from cyanobacteria grown in 99.92 % D_2O, second *(ii)*, the preparation of large quantities of uniformly labelled nucleoside 5'-triphosphates (dNTPs) obtained from fully labelled DNA or RNA by enzymatic conversion of both ribo- and deoxyribonucleotides and, third *(iii)*, the large scale synthesis of labelled oligonucleotides, realised in a specially designed PCR thermocycler that can accommodate up to 2 litres of reaction mixture.

This procedure can be easily generalised to the large scale preparation of uniformly or selectively labelled (^{13}C, ^{15}N and/or ^{2}H) double stranded DNA of almost any defined sequence.

Strategies For Radiolabelling Biological Molecules
Part II, Oligonucleotides, DNA and RNA

Martin James and Barry Kent
Ligand Development Service
Amersham Pharmacia Biotech
Cardiff Laboratories, Forest Farm, Whitchurch
UK

Historically, oligonucleotides, DNA and RNA have been radiolabelled with isotopes such as ^{32}P and ^{35}S for use in molecular biology applications. More recently there has been a demand for more sophisticated forms of labelling using a wider range of isotope for an increased number of applications, such as antisense therapies and high throughput screening.

In this presentation we will review both the historical perspective and recent advances in this field, including chemical, fermentation, enzymatic and synthetic methodologies. Emphasis will be placed on recent developments which give greater control over the choice of isotope, specificity of labelling and achievable yield.

The sulphation of synthetic heparin-like antithrombotics with pyridine·$^{35}SO_3$

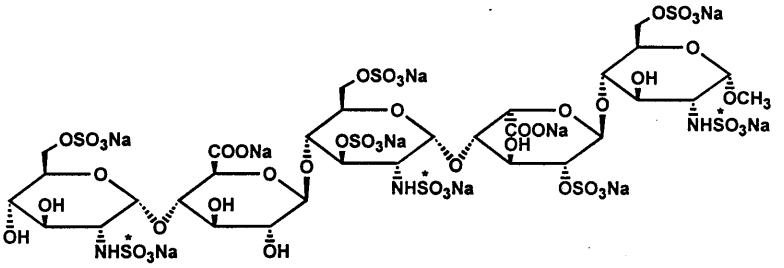
Eric Sperling, Marcel van den Heuvel, Jan Vader and Frans Kaspersen
N.V. Organon, P.O.Box 20, 5340 BH Oss, The Netherlands

Introduction

Heparin is a well known antithrombotic drug. It is purified from biological sources such as intestinal mucosa and consists of a mixture of glycosaminoglycans. The shortest fragment in the polymer with high affinity for the protease inhibitor antithrombine III (AT III) is a unique pentasaccharide fragment. The synthetic counterpart of this fragment, Org 31540/SR90107A co-developed by Organon and Sanofi[1], catalyses the AT III mediated inactivation of factor Xa but not of thrombin and is now entering clinical phase III.
In a colaboration between Organon and Sanofi simplified analogues like SanOrg 32701 and SanOrg 34006 were synthesized[1].
For preclinical development studies these synthetic heparin-like antithrombotics were needed in radiolabelled form. Although the labelling with ^{3}H or ^{14}C would be preferable from metabolic point of view, such syntheses would require more than ten reaction steps with radiolabelled materials. Moreover, for Org 31540 most of the anti-Xa activity was recovered in the urine samples of animals dosed with such a pentasaccharide indicating that no metabolic desulphation of the essential sulphate groups occurred. Labelling of these synthetic antithrombotics with ^{35}S was achieved with pyridine·$^{35}SO_3$, provided that the reagent is *i.* freshly prepared, *ii.* properly dried, *iii.* good crystalline, *iv.* only slightly coloured and *v.* of a specific activity between 0.2 and 0.4 Ci/mmol. The compounds were prepared either by specific N-sulphation or by per-O-sulphation.

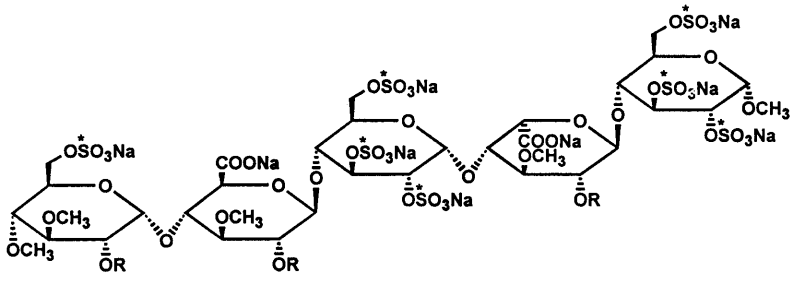
Results and Discussion

Specific N-sulphation of the partially O-sulphated precursor of Org 31540 was obtained by reaction with 5 equivalent of pyridine·$^{35}SO_3$ per NH-group in diluted aqueous $NaHCO_3$ and resulphation with another 5 equivalent of "cold" pyridine·SO_3 per NH-group. Typically 3-6 % of "high molecular ^{35}S" and 94-97 % of $Na_2{}^{35}SO_4$ were obtained after size-exclusion chromatography. Further purification by ion-exchange chromatography in order to remove undersulphated species gave 2-4 % of pure [^{35}S]-Org 31540 with a specific activity of 0.16-0.37 Ci/mmol.



[^{35}S]-Org 31540

Per-O-sulphation of the hydroxyl groups of the precursors of pentasaccharides like SanOrg 32701 and SanOrg 34006 could also be achieved with pyridine·$^{35}SO_3$. Surprisingly good sulphation was obtained only in N,N-dimethyl-formamide (DMF). Small scale "cold" experiments indicated that unlike DMF other polar aprotic solvents like N-methylpyrrolidinone and N,N-dimethylacetamide were not suitable for the sulphation at all. Initially such a "hot" O-sulphation was carried out with 1 equivalent of pyridine·$^{35}SO_3$ per OH-group at 50-60 °C and further persulphated with 5 equivalents of "cold" triethylamine·SO_3 per OH-group at 50-60 °C. Later on it was found that both the "hot" sulphation and the "cold" resulphation could be done with pyridine·SO_3 at 30 °C. Starting with 5 equivalents of good (*vide supra*) pyridine·$^{35}SO_3$ per OH-group in dry, amine-free DMF overnight at 30 °C and resulphation with another 5 equivalents of "cold" pyridine·SO_3 per OH-group again overnight at 30 °C, reproducable results with respect to the yield (10-15 % after gelfiltration, 5-10 % of final product) as well as to the specific activity (# OH groups x spec. act. py·$^{35}SO_3$ x 80 %) were obtained.



[^{35}S]-SanOrg 32701 : R = $^{35}SO_3^-$
[^{35}S]-SanOrg 34006 : R = CH_3

All compounds were prepared with ≥ 95 % radiochemical purity on radio-HPLC.
The specific activities were determined by a bioassay (anti-Xa activity) combined with liquid scintillation counting (LSC) and confirmed by HPLC combined with LSC.

References

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^{11}C-labelled Aromatics and ^{18}F- Electrophilic Fluorinating Agents as Important Prerequisites for the Development of Modern Radiopharmaceuticals

J. Steinbach

Institut für Bioanorganische und Radiopharmazeutische Chemie,

Forschungszentrum Rossendorf, D-01314 Dresden, Postfach 510119, Germany

Introduction

Originally PET (Positron Emission Tomography) was started with knowledge from classical radiochemistry, synthetic and analytical chemistry as well as nuclear physics. From these sources the base for PET became established. At that time the fundamentals for the whole technique were developed. During the last decade PET has became a tool for routine in medical diagnosis. This is closely connected with the introduction of modern whole body PET cameras and their application for cancer diagnosis. The radiochemists and radiopharmacists are forced to fulfill the requirements for daily routine in particular with regard to the introduction of GMP (Good Manufacturing Practice) to the preparation of PET-radiopharmaceuticals. Accordingly, it becomes more and more difficult to spend time doing research work. However, should there be any possibilities for doing research these efforts are directed towards applied research to label substances which could have direct impact on any medical research programme.

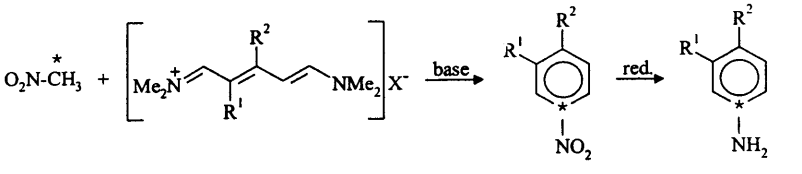
Nevertheless, from the point of view of labelling chemistry there is a lack of tools. Such tools can only be developed by systematic investigations. The share of our group to this task are two contributions:

1. The introduction of the short lived radionuclide ^{11}C into ring positions as in benzenoid and heteroaromatic compounds,
2. Electrophilic radiofluorination with no carrier added ^{18}F.

Results

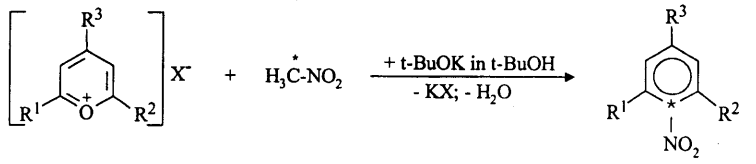
1. Ring systems are structural components of a widespread variety of naturally occuring and biologically active compounds. In numerous cases ring labelling with ^{11}C is the only method for isotopic labelling of a molecule at all or in metabolically stable positions. We have introduced a few new methods to synthesize such ^{11}C-ring-labelled compounds [1,2]:

First method: Reaction of nitro-[^{11}C]methane with suitable pentamethinium salts in the presence of a base according to Scheme 1 yields a variety of benzene derivatives:



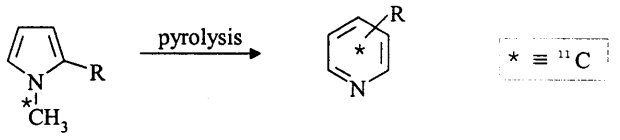
Scheme 1

Second method: Condensation of nitro-[^{11}C]methane with pyrilium salts to further benzene derivatives (Scheme 2):



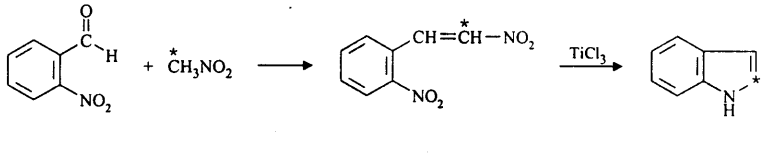
Scheme 2

Third method: Thermal rearrangement of N-[^{11}C]methyl-pyrroles at 750°C yields [^{11}C]pyridines in good yields (Scheme 3):



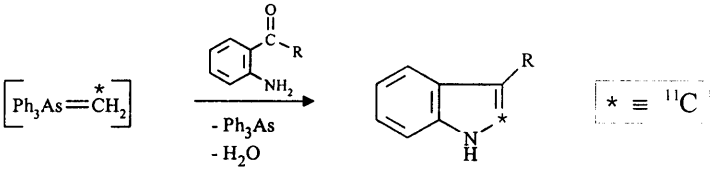
Scheme 3

Fourth method: Condensation of nitro-[^{11}C]methane with o-nitrobenzaldehyde and following reduction gives [^{11}C]indole (Scheme 4):



Scheme 4

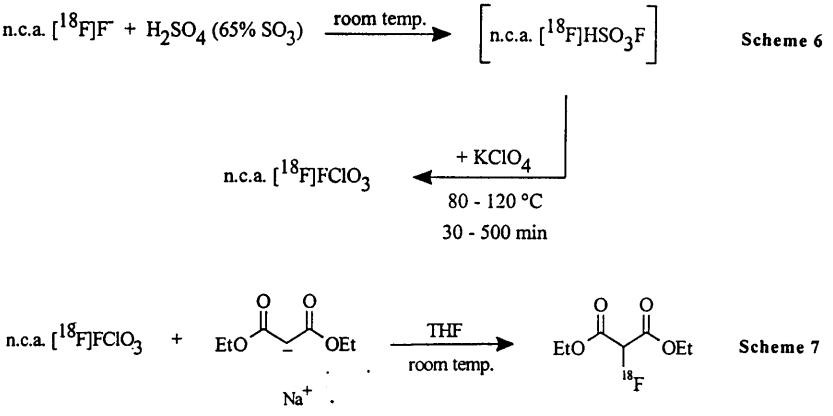
Fifth method: Reaction of the new ^{11}C-labelled substance triphenylarsonium-[^{11}C]methylide with o-amino-phenacyl compounds to various [^{11}C]indole derivatives (Scheme 5):



Scheme 5

All these methods are capable of being used for synthesis relating to the yields achievable. They differ from the derivatives which can be synthesized.

2. The electrophilic radiofluorination is the usual method for introducing ^{18}F into electron rich sites of molecules such as benzenoid rings. It it routinely used in demetallation reactions as for the synthesis 6-[^{18}F]fluoro-DOPA. Nowadays it is connected with various disadvantages: For that porpose only a low yield nuclear reaction for ^{18}F production can be applied and the resulting products have a poor specific activity. This has particular importance in the synthesis of ligands for receptors with low densities. This is a problem of general interest [3] and therefore we concentrate our efforts on developing a procedure for Umpolung of n.c.a. [^{18}F]fluoride to n.c.a. electrophilic fluorination agents. Even though the yield has been low until now it is the first example of synthesizing a n.c.a. ^{18}F labelled electophilic fluorination agent and its conversion to n.c.a. ^{18}F labelled compounds [4].



Scheme 6

Scheme 7

In this work are involved the members of the staff of the PET-Tracer group Dipl.-Chem. P. Mäding, Dr. J. Zessin, Dr. F. Füchtner, Dipl. Chem. K. Neubert and Dr. K. Chebani.

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Synthesis and Radiosynthesis for *in vivo* Imaging Using PET

Juliette Martin, Eddie Marrière, Cécile Perrio-Huard, Louisa Barré and Marie Claire Lasne*

Laboratoire de Chimie Moléculaire et Thioorganique, UMR CNRS 6507.
Université de Caen-Basse Normandie, Centre Cyceron, Institut des Sciences de la Matière et du Rayonnement, 6 Boulevard du Maréchal Juin, 14050 Caen Cedex, France
*E-mail : lasne@ismra.fr

The development of methods for introducing carbon-11 ($t_{1/2}$: 20.4 min) or fluorine-18 ($t_{1/2}$: 109.6 min) in biomolecules has been essential for the progress in understanding biochemical processes *in vivo* using Positron Emission Tomography.[1] This requires either the synthesis of new labelled precursors, the development of rapid reactions or the preparation and biological evaluation of the most active enantiomer of a given ligand. These differents aspects can be illustrated by

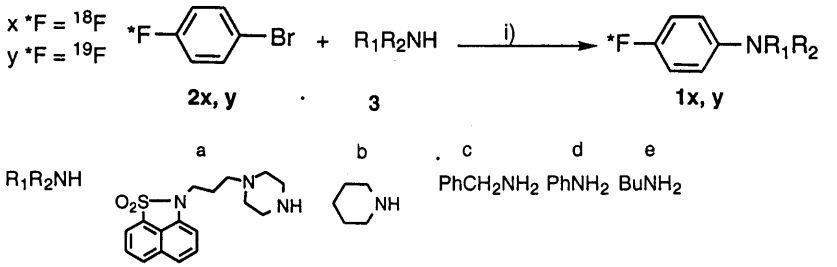
- the radiosynthesis of [^{18}F]RP 62203 **1ax,** antagonist of 5-HT_2 receptors, *via* a cross coupling reaction between 4-[^{18}F]fluorobromobenzene[2] **2x** and amine **3a** (scheme1),

- the preparation of (*R*)-(-) and (*S*)-(+)-AF-DX 384 **4** including the development of an asymmetric route to the key intermediate **5**[3] (scheme 2),

and by the one-pot preparation of amides-^{11}C **9**[4] (scheme 3).

Cross coupling reaction of 4-[^{18}F]fluorobromobenzene with amines

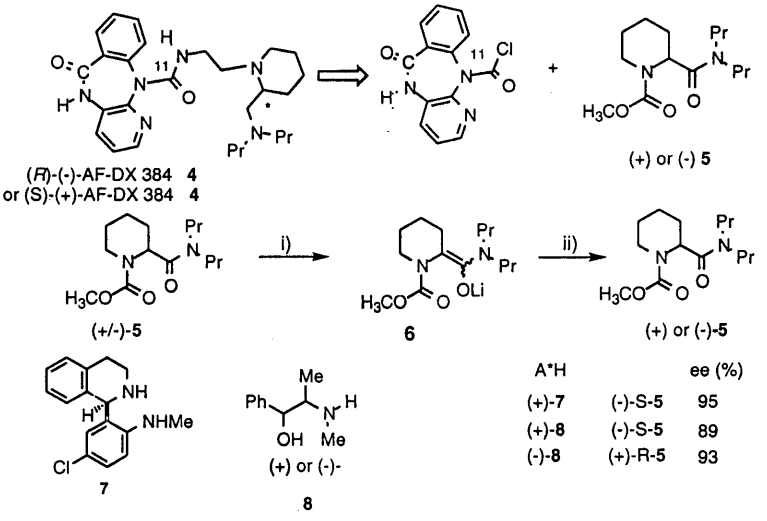
The reaction of bromobenzenes with amines in the presence of a palladium catalyst and a base has been largely studied for the last five years.[5] When amines **3a-e** were treated with 4-fluorobromobenzene **2y** in the presence of $Pd_2(dba)_3$/BINAP or $PdCl_2$/[P(o-tolyl)$_3$]$_2$ and tBuONa, for 2h, 4-fluoroanilines **1y** were obtained in moderate to good yields (**1ay**: 65%; **1by**: 57%; **1cy**: 95%; **1dy**: 96%; **1ey**: 73%). Attempts of cross coupling [^{18}F]fluorobromobenzene **2x** with primary amines **3c-e**, for 10 min using tBuONa and the same complexes [$Pd_2(dba)_3$/BINAP, $PdCl_2$[P(o-tolyl)$_3$]$_2$] or $Pd_2(dba)_3$/P(o-tolyl)$_3$ failed. However, this last catalyst [$Pd_2(dba)_3$/P(o-tolyl)$_3$] allowed the reaction of secondary amines **3a-b** with **2x** in 60-65% (corrected for decay) and 15 min reaction time. [^{18}F]RP 62203 **1ax** was prepared in 10-13% radiochemical yields (corrected for decay) from [^{18}F]fluoride (190 min, HPLC and formulation for intraveineous injection included, 99% radiochemically pure).



Scheme 1: Reaction conditions i) *t*-BuONa, toluene, 110°C, $Pd_2(dba)_3$/BINAP or $PdCl_2$[P(o-tolyl)$_3$]$_2$ or $Pd_2(dba)_3$/[P(o-tolyl)$_3$.

Synthesis and biological evaluation of (+)- and (-)-AF-DX 384.

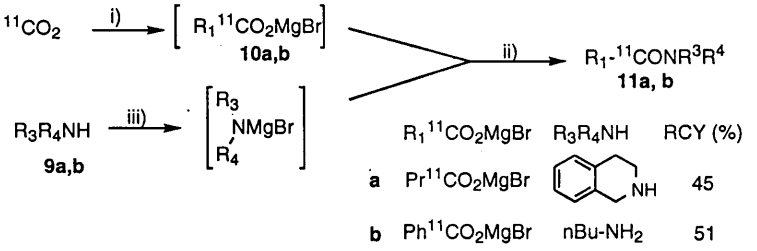
AF-DX 384 **4** is a potent and selective antagonist of muscarinic M_2 receptors. It has been synthezized[6] and labelled with carbon-11.[7] However, in the light of the fact that the spacial arrangement of the moities interacting with the receptors plays a decisive role for affinity to the receptor, both enantiomers of **4** were prepared. The *in vitro* evaluation showed that (*R*)-(-)AF-DX-384 exhibits a 23 fold higher affinity than its enantiomer (*S*)-(+). An asymmetric route to the key intermediate **5** was developed. It was based on the asymmetric protonation of the amide enolate **6** in the presence of the amines (+)-**7**, (+)-**8** or (-)-**8**.



Scheme 2: Retrosynthetic analysis of AF-DX-384 and reaction conditions i) sBuLi, LiBr, -78°C; ii) A*H then H_2O, -78°C then RT.

Rapid syntheses of amides-^{11}C

In the search for new rapid reactions, a one-pot synthesis of aliphatic amides-^{11}C was achieved by treatment of [^{11}C]- carboxymagnesium halides with amines.[4] Activation of amine **9b** as a magnesium bromide amide allowed the reaction of benzoate **10b** with a primary amine. *N*-Butyl- [1-^{11}C]benzamide **11b** was obtained in 51% radiochemical yield (decay corrected) (total synthesis time : 25 min)



Scheme 3: Synthesis of [^{11}C]amides i) PrMgBr, THF, 0°C, 3 min; ii) THF, -70°C, 5 min; iii) PrMgBr, THF, -70°C, 15 min.

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THE BIOSYNTHESIS OF THE FLUORINATED NATURAL PRODUCTS FROM *STREPTOMYCES CATTLEYA*. DEUTERIUM EXCHANGE INTO THE FLUOROMETABOLITES FROM THE MEDIUM

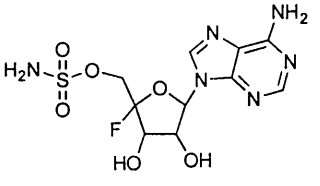
Muhammad R Amin[1], David B. Harper*[2] and David O'Hagan*[1]

[1]Dept. Of Chemistry, Science Labs., University of Durham, South Road, Durham, UK, DH1 3LE.
[2]Microbial Biochemistry Section, Department of Food Science, The Queen's University of Belfast, Newforge Lane, Belfast, UK, BT9 5PX.

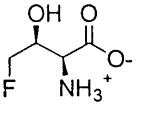
Summary: $^{19}F\{^1H\}$-NMR has been used directly to observe deuterium induced isotope shifts reporting the incorporation of deuterium atoms from ^2H_2O, into fluoroacetate and 4-fluorothreonine during their biosynthesis by *Streptomyces cattleya*. The different populations of deuterium labelled fluorometabolites are readily discerned by $^{19}F\{^1H\}$-NMR.

Key words: Fluoroacetate, 4-fluorothreonine, *Streptomyces cattleya*, deuterium induced ^{19}F-NMR shifts.

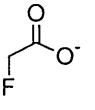
Fluorinated natural products are extremely rare, and Nature has not generated a wide spread ability to form carbon-fluorine bonds [1]. The most common fluorinated natural product is the toxin fluoroacetate which has been found in over 40 plant species. Only two bacteria have been identified which elaborate organo-fluorine compounds. *Streptomyces calvus* elaborates the antibiotic nucleocidin [2] and *Streptomyces cattleya* is particularly active in its ability to generate fluoroacetate and 4-fluorothreonine [3]. Both of these micro-organisms clearly posses an enzyme capable of catalysing formation of the C-F bond.



nucleocidin



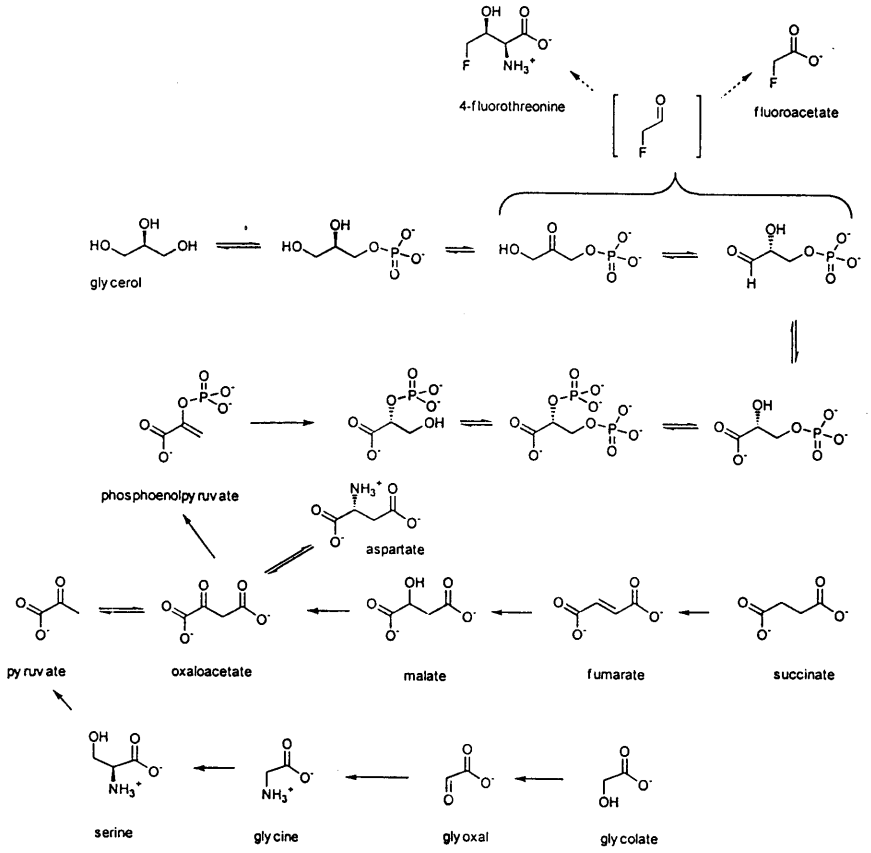
4-fluorothreonine



fluoroacetate

Investigations into the biosynthesis of fluoroacetate and 4-fluorothreonine in *S. cattleya* have been greatly facilitated by the presence of the fluorine atom in the natural product as fluorine-19 is a NMR sensitive nucleus (I = ½) and incorporations of carbon-13 and deuterium into these metabolites in feeding experiments are readily diagnosed from ^{13}C-^{19}F and ^2H-^{19}F couplings and induced isotope shifts. We have recently demonstrated [4, 5] the incorporation of a range of carbon-13 labelled

precursors such as [2-^{13}C]-, and [1,2-$^{13}C_2$]-glycine, [1-^{13}C]-, [1-^{13}C]- and [3-^{13}C]- pyruvate, [3-^{13}C]-serine, into the fluorometabolites and the regioselectivity of their incorporations into fluoroacetate and 4-fluorothreonine was revealed by $^{1}J_{13C\text{-}19F}$ and $^{2}J_{13C\text{-}19F}$ couplings in the resultant ^{19}F-NMR spectra. That study revealed that glycine was converted to L-serine and then to pyruvate and that pyruvate was channelled into the glycolytic pathway, after carboxylation to oxaloacetate to prime a conversion to phosphoenolpyruvate as indicated in Scheme1. There is substantial evidence that the glycolytic pathway provides the precursors to the fluorometabolites particularly as we [6] and a Japanese group [7] have both demonstrated the efficient incorporation of glycerol into the fluoroacetate and 4-fluorothreonine and our current working hypothesis on the pathway to the fluorometabolites from *S. cattleya* is outlined in Scheme 1.



Scheme 1

In this paper we illustrate the power of ^{19}F{^{1}H}-NMR in identifying the level of incorporation and regiospecificity of deuterium labelling into fluoroacetate and particularly into 4-fluorothreonine.

During the biosynthetic process certain sites become labile to enolisation and isotope is lost due to exchange with the aqueous medium. For example we have already reported [4, 6] the incorporations of [$^2H_2$]-glycine, [$^2H_3$]-aspartate and (R)-[1-$^2H_2$]-glycerol into fluoroacetate and 4-fluorothreonine. Despite significant levels of exchange from [$^2H_2$]-glycine and [$^2H_3$]-aspartate the low levels of deuterium incorporation allowed us to conclude that both of the methylene hydrogens of glycine and of C-3 of aspartate can contribute both methylene hydrogens of the fluoromethyl group. This reinforces our working hypothesis and is consistent with the conclusions set out in Scheme 1. In order to explore deuterium incorporations further we have now studied the reverse issue of deuterium incorporation by exchange from ^2H_2O into the fluorometabolites of *S. cattleya*. Such incorporations could arise by enolisation, but also from co-factor reduction (eg NADPH) where the cofactor has become labelled during metabolism.

Results and discussion

Two cultures of *S. cattleya* were innoculated from a mature culture in a manner previously described [4] and the medium was supplemented with 10% ^2H_2O and 2mM fluoride. Fluorometabolite biosynthesis was allowed to progress for a 6 day period after which time the cells were centrifuged and the supernatant concentrated and analysed by $^{19}F\{^1H\}$-NMR. Proton decoupling was applied to

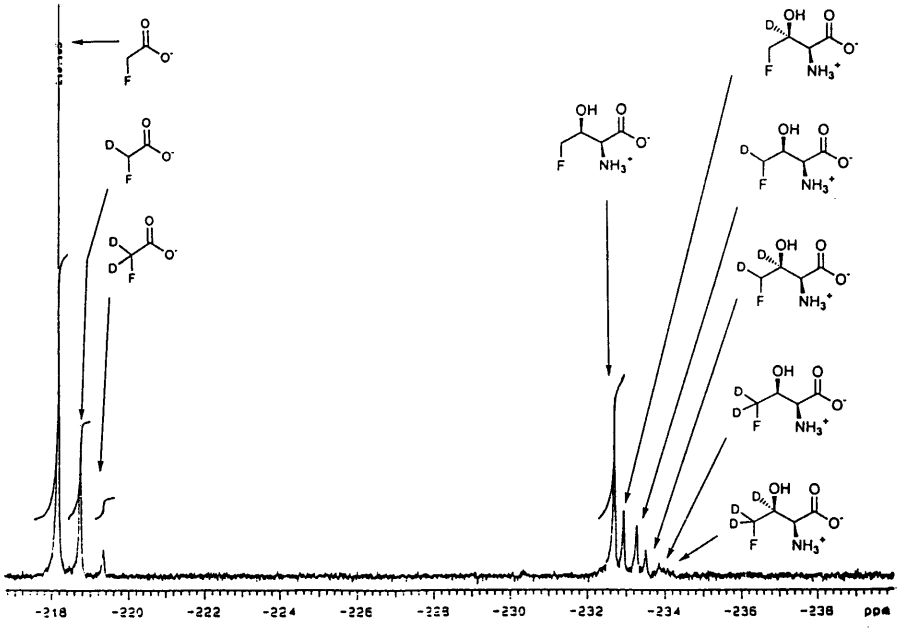


Figure 1 $^{19}F\{^1H\}$-NMR spectrum of fluoroacetate and 4-fluorothronine in the supernatant of *S. cattleya* after incubation in 20% ^2H_2O. The different labelled populations are assigned.

remove ^{19}F-^{1}H couplings and simplify the resultant spectrum. Figure 1 illustrates the resultant $^{19}F\{^{1}H\}$-NMR spectrum from the experiment. It is clear from the induced isotope shifts that a significant level of deuterium incorporation has occurred into both fluoroacetate and 4-fluorothreonine during the biosynthesis. The fluoroacetate signal (-218.17ppm) is accompanied by two shifted signals to lower frequency. These signals arise due to populations of fluoroacetate molecules carrying one deuterium (-218.75ppm, β-shift 0.58ppm) and two deuteriums (-219.34ppm, β-shift 1.17ppm) respectively. The 4-fluothreonine signal (-232.7ppm) has four additional upfield shifted signals associated with it indicating at least five different populations of deuterium labelled amino acid. The fluoromethyl group of 4-fluorothreonine can accommodate one or two deuterium atoms in a similar manner to fluoroacetate. Additionally however deuterium incorporation into C-3 of 4-fluorothreonine is readily observable due to an induced γ-shift of ~0.3ppm. Thus these four labelled populations arise from a γ shift, a β shift, a β + γ shift, a 2 x β shift and a low intensity 2 x β + γ shift. Thus all compliments of label into C-3 and C-4 of 4-fluorothreonine are represented and can be assigned. Clearly deuterium is likely to become incorporated into the α-carbon at C-2 of 4-fluorothreonine in this experiment, however the resultant δ-shift on the ^{19}F-signal is too small to resolve in the spectrum. The various populations of labelled molecules are assigned in Figure 1.
The ability to identify deuterium incorporation directly by $^{19}F\{^{1}H\}$-NMR into C-3 of 4-fluorothreonine in such a straightforward manner will allows us now to explore the origin of this hydrogen atom appropriate deuterium labelled precursors.

Acknowledgements: We thank BNFL Company Research Laboratories for support to this project and Ian H McKeag of the University of Durham for recording the $^{19}F\{^{1}H\}$-NMR spectra.

Experimental

Streptomyces cattleya NRRL8057 was cultured in 2mM fluoride as previously described [4]. $^{19}F\{^{1}H\}$-NMR spectra were recorded on a Bruker Spectrospin AMX-500 instrument operating at 471.54MHz and with a proton decoupling frequency of 500.14MHz.

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Use of Isotope-labelling in NMR Studies of Protein Ligand Interactions

J. Feeney
National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK.

The combination of isotopic labelling of proteins and their binding ligands (using ^{2}H, ^{3}H, ^{13}C, ^{15}N) with modern multidimensional NMR techniques forms a central part of the strategy for studying protein-ligand interactions by NMR. Proteins larger than 15 kDa require $^{13}C/^{15}N$ labelling approaches to allow simplification of their NMR spectra such that complete sets of resonance assignments and NOE (nuclear Overhauser effect) data can be obtained. This information is needed to extract the distance and torsion angle constraints required for the structural determinations. Isotope editing and filtering experiments allow NOEs for certain nuclei to be selectively detected. Isotopically labelled ligands are also useful for experiments involving direct or indirect detection of the heteronuclear label: these approaches provide simplified spectra which assist in determining protonation states, detecting multiple conformations and measuring dynamic processes within the complexes. We have been applying these methods to study several protein-ligand complexes.

Our studies have used mainly a combination of high resolution NMR techniques, molecular modelling and biochemical approaches. We are particularly interested in drug-receptor complexes where characterising the interactions has a practical significance since it could assist in rational drug design. We have been trying to understand the molecular basis for binding specificity in several systems in solution. We have carried out structural determinations of complexes of dihydrofolate reductase formed with antifolate drugs and characterised specific interactions, conformational mixtures and protein/ligand correlated dynamic processes. Antifolate drugs act by inhibiting the enzyme in parasitic or malignant cells (typical examples are the antibacterial trimethoprim and the anticancer methotrexate). The value of $^{15}N/^{1}H$ and $^{13}C/^{1}H$ HSQC experiments for studies of dynamic processes has been explored.

We have also been investigating the use of ^{15}N chemical shifts to probe site specific Ca^{2+} binding to calmodulin and related calcium binding proteins. We have also carried out structural determinations of the protein tissue inhibitor of metalloproteinases (TIMP-2) and defined its interaction surface in the complex with a 19 kDa catalytic domain from stromelysin by monitoring the $^{1}H/^{15}N$ chemical shift perturbations which accompany complex formation. Examples of the NMR determined information from these various systems will be considered.

^{14}C- Labelling of NVP RAD001 - A New Rapamycin Derivative [1]

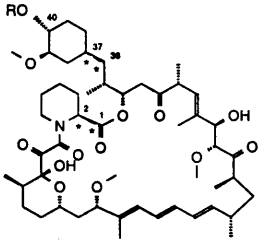
Th. Moenius [1]*, R. Voges [1]*, M. Mahnke[2], P. Burtscher[1], Y. Metz[1] and Ch. Guenat[3]

[1] NOVARTIS PHARMA AG., Preclinical Safety, CH-4002 Basel, Switzerland.

[2] NOVARTIS PHARMA AG., Biomolecules Production, CH-4002 Basel, Switzerland.

[3] NOVARTIS PHARMA AG., CH-4002 Basel, Switzerland.

There is an increasing interest in the immunosuppressant rapamycin **1a**, a macrolide produced by *Streptomyces hygroscopicus* [2,3]. In a preclinical program NVP RAD001 **1b** (C(40)-O-(2-hydroxyethyl)-rapamycin) was designed to overcome difficulties observed in drug development of rapamycin **1a**.



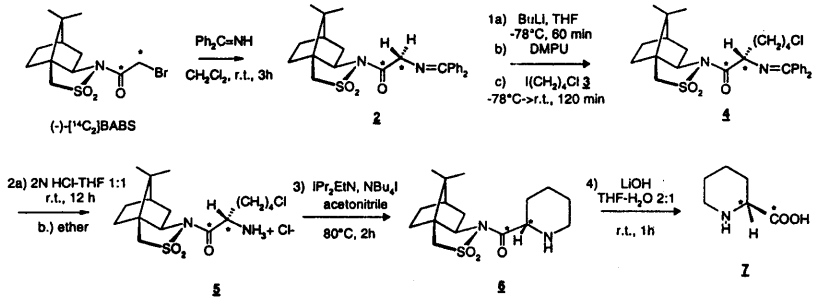
R = H **1a** (Rapamycin)

R = HO-CH_2-CH_2- **1b** (NVP RAD001)

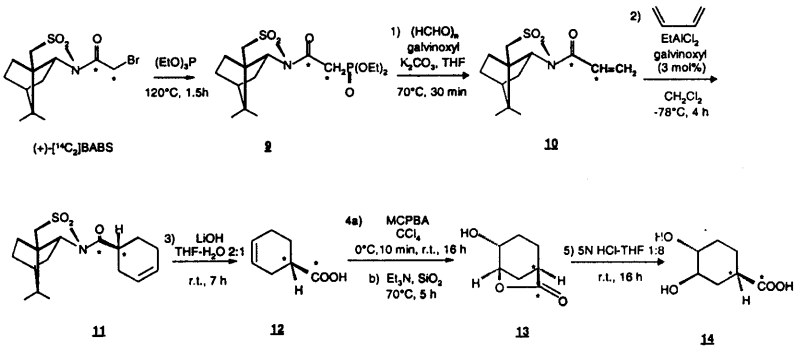
This short communication describes the carbon-14 labelling of rapamycin.

[$^{14}C_2$]-L-Pipecolic **7** acid and (1R,3R,4R)-3,4-dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid **14** ([^{14}C]DHCCA) synthesized in effective enantioselective approaches starting from (-)- and (+)-[$^{14}C_2$]BABS (scheme 1 and 2), respectively, proved to be highly suitable precursors [4,5] for the fermentative synthesis of carbon-14 labelled rapamycin **1a**.

Scheme 1: Enantioselective Synthesis of [$^{14}C_2$]-L-Pipecolic acid



Scheme 2: Enantioselective Synthesis of (1R,3R,4R)-3,4-dihydroxy[1,7-$^{14}C_2$]cyclohexane carboxylic acid ([^{14}C]DHCCA)



Based on *Streptomyces hygroscopicus* a fermentation process was optimized, which is characterized by good reproducibility, high radiochemical purity of the crude product (> 60%), excellent radiochemical yield (> 10%) and nearly complete recovery of the radioactivity employed (>90%). Combined addition of both precursors afforded C-14 labelled rapamycin of a specific activity > 100 mCi/mmol.

Mass spectroscopy (ESI) gave evidence that 62 % of the radioactivity derived from the [$^{14}C_2$]-L-pipecolic acid, 38 % from the [$^{14}C_2$]DHCCA.

In a two-step sequence [$^{14}C_4$]rapamycin **1a** was converted by C(40)-O-alkylation using monosilylated ethylenglycol triflate (N,N,diisopropylethylamine, toluene/dimethoxyethane, 50°C, 3h) and final deprotection (acetic acid, 20°C, 60 min.) to [$^{14}C_4$]NVP RAD001 **1b**.

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LOCAL AND GLOBAL ^{14}C- CONCENTRATIONS

Heinz Hugo Loosli
Physics Institute, University of Berne, Sidlerstrasse 5, CH- 3012 Berne

ABSTRACT

On the regional scale, increased ^{14}C activities in plants from the vicinity of nuclear and industrial installations are relatively easy detectable; increased activities are measured to be up to $\Delta^{14}C \simeq +400$ ‰ in Switzerland. Additional doses are the consequences for individuals, which, however, are small compared to the natural ^{14}C dose of about 13 μSv per year.

On the global scale, natural production of ^{14}C and the remaining activity from nuclear bomb tests are the dominant sources. Compared to these levels, the atmospheric ^{14}C activity released from nuclear installations is of minor importance. For understanding the global carbon cycle and for calibration of the circulation and dilution models the natural and man made ^{14}C activity is, however, very important and useful.

Key words: natural ^{14}C activity; ^{14}C -emissions; local dispersion; radiation dose

I. INTRODUCTION

^{14}C is a very unique isotope: it is well known to be applied for dating in archaeology, quartenary geology, hydrology, oceanography and other fields of earth science.

It is also used as a label for biological, pharmaceutical and other tracer investigations. On a global scale it is applied for the calibration of models describing quantitatively the carbon cycle, which are needed to predict future climate change. Some of these regional and global applications are described briefly in this contribution. Basic information however is found in table 1.

TABLE 1: BASIC INFORMATION ON ^{14}C - CONCENTRATION

Natural production in the atmosphere:	^{14}N (n, p) ^{14}C
Equilibrium activity in the atmosphere: corresponding to natural specific activity:	~ 0.04 Bq/m^3 227 Bq/kgC
Activity in human body:	~ 3000 Bq
Average natural dose:	~ 13 μSv/y
Atomic bomb testing (activity in atmosphere):	
• increase in 1962	$\Delta^{14}C \simeq 1000$ ‰
• increase today above preindustrial level	$\Delta^{14}C = 110$ ‰
Burning of fossil fuel (activity in atmosphere)	
• estimated decrease today	$\Delta^{14}C \simeq -50$ ‰

II. LOCALLY INCREASED ^{14}C - CONCENTRATIONS

Plants accumulate artificial ^{14}C by assimilation if it is emitted in the form of CO_2. ^{14}C - measurements in tree leaves therefore offer a sensitive possibility to determine the emission of artificial ^{14}C and to compare measured results with model calculations.

Since about 20 years ^{14}C activities have been measured in the environment of Swiss nuclear installations. Results are published in KUeR reports [1] and in BAG reports [2]. It could e. g. be shown that emissions by boiling water reactors of about $2 \cdot 10^{11}$ Bq per year increase the ^{14}C level in tree leaves by about 100 ‰ ($\Delta^{14}C$) in the critical distance with maximal concentration (emission height is about 100m). The comparison with Gaussian dispersion models show an agreement within about a factor of 2. Calculated hypothetical doses are below 1 μSv per year [3] and therefore negligible.

Since 1994 ^{14}C activities in the environment of the incinerator of CIBA respectively NOVARTIS have been measured in Basle. This company is interested to determine possible increased immissions and corresponding doses. Yearly results are published in [2]. As example the results measured in tree leaves which have grown in 1997 are plotted in figure 1. The area of the circles is proportional to the $\Delta^{14}C$ value which is in the maximum about 400 ‰. The agreement between observed wind directions during burning of waste with ^{14}C activities and observed sectors of increased ^{14}C levels is good, also the comparison of the ^{14}C values with model calculations. For the dilution a short term dispersion factor of 10^{-4} s/m^3 has been assumed. Again calculated additional doses are almost 1000 times smaller than natural external and internal doses and also smaller than their variability. An open problem at present is the question how much carbon in a newly formed leave is assimilated during the considered growing period and how much originates from previous years. In spring when the structure of the leave is constructed, this memory- effect may be larger than in the yearly average.

III. GLOBAL ^{14}C ACTIVITIES

Information about global carbon inventories and fluxes can be found in [4], [5] and [6]. Such fluxes could e. g. be quantified by measuring time series of the ^{14}C activity in atmospheric CO_2 or in tree leaves at reference stations. Early observations represent the equilibrium of naturally produced ^{14}C whereas measurements in the last 35 years show the atmosphere-ocean exchange of CO_2 and $^{14}CO_2$, diluting the bomb produced atmospheric ^{14}C excess. As example Figure 2 illustrates the decrease of the bomb produced $\Delta^{14}C$ values in tree leaves at Swiss reference stations. All $\Delta^{14}C$ values mentioned in chapter II are net values above these yearly reference levels.

In [6] it was demonstrated that on a global scale two of the ^{14}C -activity sources dominate; natural and bomb production. ^{14}C -activities produced by nuclear power plants , by reprocessing plants or by nuclear industry are much smaller [7] although locally increased levels may be observed.

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Fig. 1: Net $\Delta^{14}C$-Values, Autumn 1997

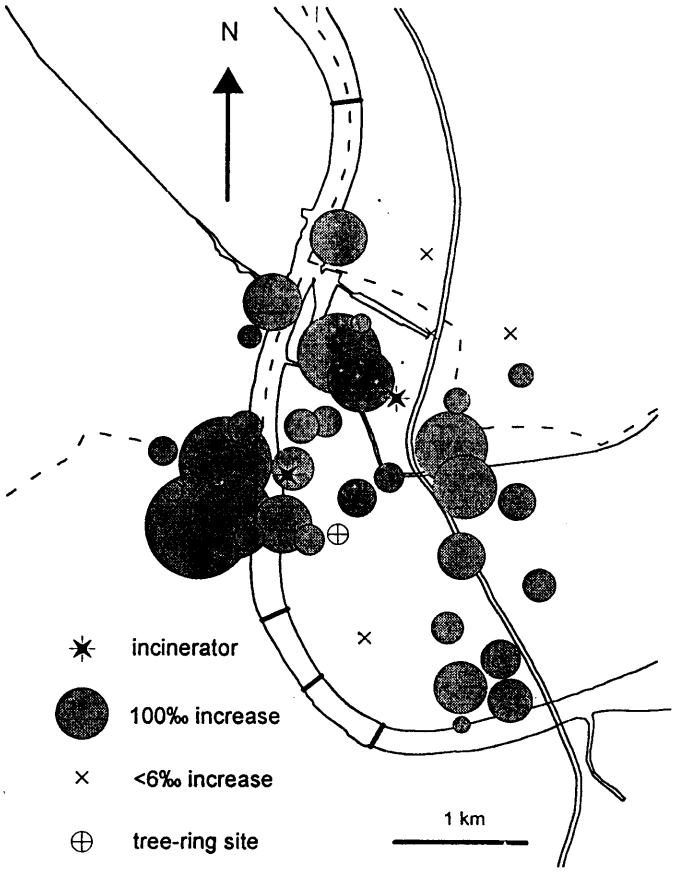
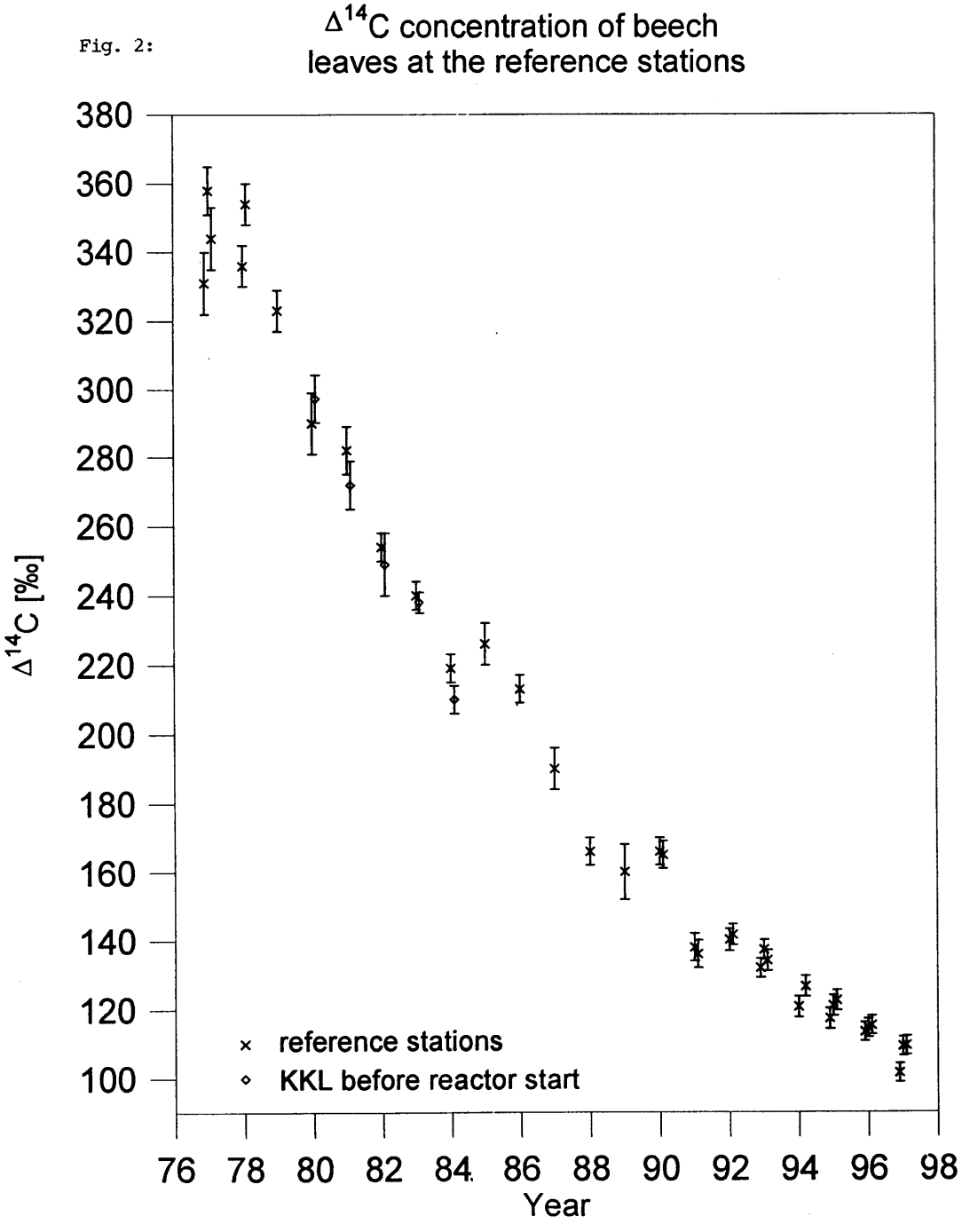


Fig. 2:



Some Applications of ^{14}C-Labelling in Ecological Research

Miroslav Matucha
Isotope Laboratory of the Institute of Experimental Botany
Academy of Sciences of the Czech Republic, Prague

The role of carbon, as a basic element of all organic compounds, predeterminates the use of the isotope ^{14}C in pathway and fate studies of xenobiotics, especially of pollutants and pesticides in the environment. We intended to elucidate two problems: (i) contribution of volatile chlorinated hydrocarbons (e.g., tetrachloroethene and methylchloroform) to forest decline and (ii) microbial degradation of PCBs.

[1,2-^{14}C]trichloroacetic acid (TCA) and [ring-^{14}C]PCB congener No. 11 and 77 were synthesized /1,2/, TCA as resulting product of the atmospheric photooxidation of above named solvents and also as an indicator of secondary air pollutants affecting forest health, and the two toxic PCB congeners for investigation of biodegradation and fate of PCBs in soil.

[1,2-^{14}C]TCA helped to elucidate the pathway and uptake of TCA from atmosphere via rain water into soil, and through roots into spruce needles. The study resulted in the finding that the main route TCA enters the spruce trees is through roots and not as a direct sorption by needles /3/.

By means of [ring-^{14}C]PCBs it was found that the main factor effecting the biodegradation rate is the soil itself - its sorption capability and organic matter content /4/. On the other hand, when PCB degradation was studied in liquid media, the main factor involved was volatilization /5/.

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Isotopically Labelled Humic Acids for Heavy Metal Complexation

M. Bubner, S. Pompe, M. Meyer, K. H. Heise, H. Nitsche
Forschungszentrum Rossendorf e. V., Institute of Radiochemistry,
P. O. Box 510119, D-01314 Dresden, Germany

Summary

Humic acids influences the speciation of actinide ions and their migration and immobilization in natural systems. We used synthetic humic acid model substances for the investigation of these very complex processes. Humic acid functionality models are synthesized from reducing sugars and ^{14}C-labelled α-amino acids and characterized by a variety of physical-chemical and analytical methods. The labelled humic acid models have functional properties comparable to those of natural humic acids.

Key Words: ^{14}C-labelled humic acids, ^{14}C-labelled melanoidins

Introduction

Humic acids (HA) are known to react with other organic substances and inorganic ions and incorporate them into their molecule. We investigate the reaction of actinide ions, especially UO_2^{2+} ions, with HA and their migration and immobilization behaviour in natural terrestrial and aquifer systems /1,2,3/. Knowledge about this enables us to develop strategies for risk assessment for the long-term safety in the regions of the former uranium mining in Saxony and Thuringia, Germany.

Our objective is to answer the following questions: Which humic acid functional groups mainly influence the complexation behaviour of HA, and what is the fate of HA during the interaction of humic-acid-metal-complexes with natural and terrestrial material.

The composition and functionality of natural HA strongly depends on their origin. Therefore it is difficult to describe natural HA and to isolate natural HA with a specific functionality. In order to study the behaviour of HA in different compartments of the environment, it would be advantageous to label the humic material with radionuclides. One possibility to introduce a radioactive label is a chemical derivatization of functional groups using a radioactive reagent.

However, the derivatization of functional groups may alter the functionality of the HA. Often such derivatives are not sufficiently stable for long-time experiments /4/. We, therefore, develop synthetic HA model substances with varied functional groups and group concentration /5/. These compounds also provide the possibility for stable radioactive labelling. Our synthetic ^{14}C-labelled HA model substances are the alkali-soluble and acid-insoluble melanoidins synthesized by the Maillard reaction /6/ from reducing sugars and selected ^{14}C-labelled α-amino acids.

Experiments

We synthesized ^{14}C-labelled model HA according Pompe et al. /7/ from xylose and ^{14}C-labelled α-amino acids, e. g., glycine and phenylalanine or glutamic acid, respectively. The alkali-soluble and acid-insoluble components of the resulting [^{14}C]melanoidins were isolated, dialysed and lyophilized. These HA model substances were characterized by elemental analysis and determination of functional groups (carboxylic groups and phenolic hydroxyl groups). The results are shown in Table 1. They agree with the data obtained with non-labelled HA model substances.

Tab. 1: Humic acid model substances from melanoidin reaction

No.	Starting material	Synthesis product		
	Amino acid	Yield [%^{14}C]	COOH [meq/g]	phen.OH [meq/g]
1	[U-^{14}C]phenylalanine, glycine	12	1.0	2.3
2	phenylalanine, [2-^{14}C]glycine	30	1.0	2.3
3	[1-^{14}C]glutamic acid	0	4.1	2.3
4	[U-^{14}C]glutamic acid	5	4.1	2.3

To determine the influence of phenolic OH groups on HA complexation behaviour, the functionality of these HA models was modified by subsequent treatment with hot alkali and by blocking the phenolic hydroxyl by methylation. The results are shown in Table 2.

Tab. 2: Humic acid model substances from modification of [^{14}C]melanoidins

No.	Precursor melanoidin (see table 1)	subsequent treatment	COOH [meq/g]	phen. OH [meq/g]
5	No. 1 and No. 2	1 N NaOH, 100°C	2.0	1.7
6	No. 1 and No. 2	CH_2N_2; 1 N NaOH, 100°C	2.0	1.0

The radiochemical yield depends on the reaction conditions. In all cases, the C-1 carboxylic group of the amino acids is eliminated during the melanoidin formation and the labelled intermediates and by-products of low molecular weight form also melanoidins during further processing. The yields are limited by the formation of insoluble humine-like substances.

The amount of amino acids that was inserted into the resulting labelled melanoidin was calculated from the resulting specific radioactivity and from the nitrogen content of the products.

Conclusion

Humic acid model substances can be isotopically labelled within the molecular backbone without damaging the HA functionality. These HA will be used to investigate the HA interaction behaviour with actinide ions such as, e. g., the sorption behaviour of actinide ions on geological materials in the presence of HA.

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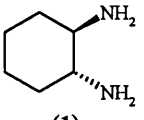
The synthesis of (*1R,2R*)-(-)-1,2-Diamino[1,2-^{14}C]cyclohexane

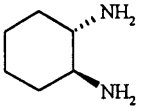
Geoffrey T. Woolley

Custom Labelling and Special Synthesis Team, Nycomed Amersham plc, Forest Farm Estate, Whitchurch, Cardiff CF4 7YT, Wales.

Both enantiomers of *trans*-1,2-diaminocyclohexane (**1**) and (**2**) form extremely useful and stable metal complexes. Some of these complexes have the potential to act as chemotherapeutic agents.[1] In addition to their medicinal applications some of these complexes have also been used in the asymmmetric hydroxylation of *trans*-alkenes,[2] and for the asymmetric epoxidation of *cis*-alkenes.[3]



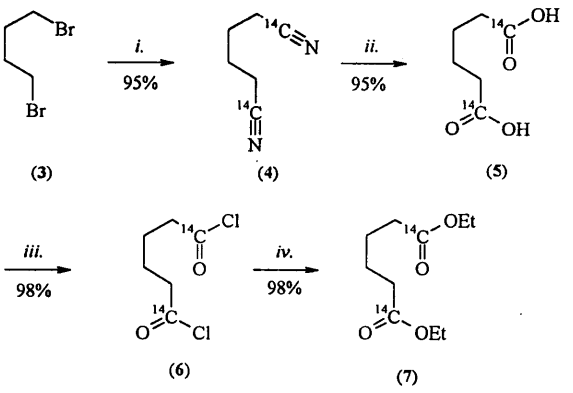
(**1**)



(**2**)

During the synthesis of a ^{14}C labelled custom preparation undertaken in our group, we found it necessary to prepare a large amount (several hundred millicuries) of (*1R,2R*)-(-)-1,2-diamino[1,2-^{14}C]cyclohexane (**17**). This was ultimately achieved by an efficient and reproducible synthesis from potassium [^{14}C]cyanide, a readily available source of ^{14}C.

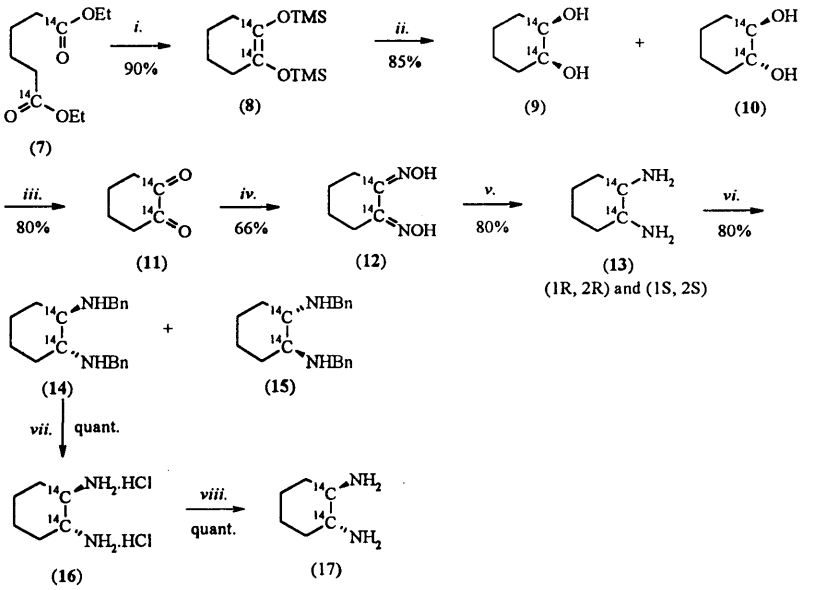
Thus 1,4-dibromobutane (**3**) on treatment with potassium [^{14}C]cyanide gave [1,6-^{14}C]adiponitrile (**4**) in 95% yield. (**Scheme 1**). [1,6-^{14}C]Adiponitrile (**4**) was hydrolysed using concentrated potassium hydroxide solution to give [1,6-^{14}C]adipic acid (**5**), once again in 95% yield. [1,6-^{14}C]Adipic acid (**5**) was treated with oxalyl chloride, in the presence of catalytic DMF, to give [1,6-^{14}C]adipoyl chloride (**6**) which was converted to diethyl [1,6-^{14}C]adipate (**7**) in 96% yield.



Reagents: *i.* $K^{14}CN$, EtOH, water, Δ. *ii.* KOH, water, Δ. *iii.* $(COCl)_2$, CH_2Cl_2. *iv.* EtOH.

Scheme 1

Diethyl [1,6-^{14}C]adipate (7) was cyclised to bis(trimethylsilyl)[1,2-^{14}C]cyclohexene-1,2-diether (**8**) in 90% yield using sodium and chlorotrimethylsilane as developed by Schrapler and Ruhlmann.[4] (**Scheme 2**). Reductive hydrolysis of the TMS diether (**8**) with sodium borohydride in aqueous ethanol gave a mixture of [1,2-^{14}C]diols (**9**) and (**10**) in 85% yield. A modified Swern oxidation of this mixture using trifluoroacetic anhydride and triethylamine in DMSO gave 1,2-[1,2-^{14}C]cyclohexanedione (**11**) in 80% yield.[5] Conversion of the [1,2-^{14}C]dione (**11**) into (+/-)-*trans*-1,2-diamino[1,2-^{14}C]cyclohexane (**13**) was achieved *via* the [1,2-^{14}C]dioxime (**12**). [1,2-^{14}C]Dioxime (**12**) was formed in 66% yield by the treatment of the [1,2-^{14}C]dione (**11**) with hydroxylamine.[6] Reduction of [1,2-^{14}C]dioxime (**12**) using sodium in ethanol gave (+/-)-*trans*-1,2-diamino[1,2-^{14}C]cyclohexane (**13**) in 80% yield.[7] This whole sequence represents an overall conversion of potassium [^{14}C]cyanide to (+/-)-*trans*-1,2-diamino[1,2-^{14}C]cyclohexane (**13**) in 28% yield.



Reagents: i. Na, TMS-Cl, toluene, Δ. *ii.* $NaBH_4$, EtOH, water. *iii.* TFAA, TEA, DMSO, -60°C. *iv.* $NH_2OH.HCl$, KOH, water, 0°C. *v.* Na, EtOH, 80°C. *vi.* PhCHO, EtOH; $NaBH_4$. vii. $Pd(OH)_2/C$, H_2, MeOH, HCl, 40°C. *viii.* 35% KOH.

Scheme 2

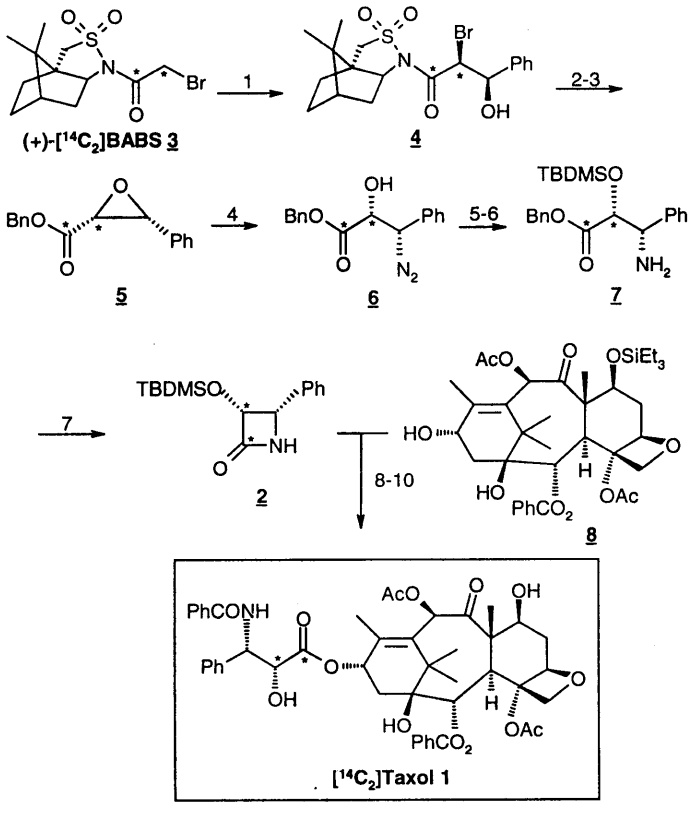
Resolution of (+/-)-*trans*-1,2-diamino[1,2-^{14}C]cyclohexane (**13**) was achieved by formation of the dibenzyl derivatives. Thus (+/-)-*trans*-1,2-diamino[1,2-^{14}C]cyclohexane (**13**) was treated with benzaldehyde and the resulting diimines reduced *in situ* with sodium borohydride to give N,N'-dibenzyl-1,2-diamino[1,2-^{14}C]cyclohexanes (**14**) and (**15**) in 80% yield. Compounds (**14**) and (**15**) were then separated using chiral HPLC. N,N'-Dibenzyl-1,2-diamino[1,2-^{14}C]cyclohexane (**14**) was deprotected using hydrogen and Pearlman's catalyst to give the relatively stable dihydrochloride salt (**16**) in quantitative yield. Treatment of the dihydrochloride (**16**) with 35% aqueous potassium hydroxide gave (*1R,2R*)-(-)-1,2-diamino[1,2-^{14}C]cyclohexane (**17**) once again in quantitative yield. This represents an overall theoretical yield of 80% for the resolution.

Stereoselective Synthesis of [sc-$^{14}C_2$]Taxol

I. Rodriguez and R. Voges

Preclinical Safety-Drug Metabolism Depatment, Isotope Section

Novartis Pharma AG, CH 4002 Basel, Switzerland



1a) $TiCl_4$, N-Ethylpiperidine, CH_2Cl_2, -78°C; 1b) PhCHO, 10 min.; 2) K_2CO_3, DMF, H_2O cat., rt.; 3) BnOLi, THF, -10°C; 4) NaN_3, HCO_2Me, MeOH:H_2O, 8:1, 50°C; 5) TBDMSOTf, 2,6-lutidine, CH_2Cl_2, 0°C; 6) Ph_3P, THF:H_2O, 8:1, 50°C, 2h; 7) *tert*-BuMgBr, ether, -10°C, 2h; 8) PhCOCl, Et_3N, CH_2Cl_2; 9) nBuLi, THF, -40°C; 10) HF-Py, 0°C

The diterpenoid Taxol **1** still continues to provide significant benefits in the clinical treatment of breast and ovarian cancers [1]. We were asked to supply carbon-14 labelled Taxol to support pharmacokinetic investigations. For this purpose the label of the molecule was placed into the pharmacologically essential (2R,3S)-phenylisoserine side chain by stereoselective synthesis of the β-lactame **2** starting from the internally developed chiral bromoacetate synthon (+)-[$^{14}C_2$]BABS **3** [2].

The stereoselective aldol reaction of the titanium enolate of (+)-[$^{14}C_2$]BABS **3** and benzaldehyde was performed to generate the syn-α-bromo aldol **4** with excellent enantioselectivity after recrystallization (82%, de > 99%). Intramolecular epoxide formation by treatment with potassium carbonate followed by cleavage of the auxiliary with lithium benzoxide afforded (2R,3R)-epoxy benzyl ester **5**. Regiospecific opening of the epoxide with sodium azide (→ **6**), O-silylation with *tert*-butyldimethylsilyl triflate and reduction of the azide group with triphenylphosphine produced the (2R,3S)-O-*tert*-butyldimethylsilyl-phenylisoserinate **7**. Cyclization to β-lactame **2** was accomplished on reacting **7** with *tert*-butylmagnesium chloride in ether. Attachement of **2** was performed according to literature procedures [3-4]. Activation of the lactame with benzoylchloride, reaction of the resulting N-benzoyl lactame with the deprotonated allylic hydroxy group of 7-TES-baccatine III **8**, and finally, desilylation with HF-pyridine afforded the labelled Taxol **1**.

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(S)- [^{14}C](DUAL: Stereoselective synthesis and use of chiral phases

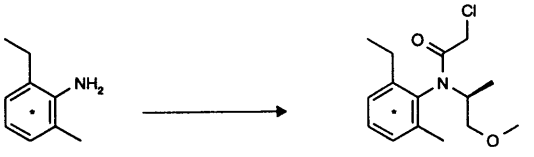
M. Respondek, F. Spindler, Novartis Crop Protection AG, Basel, Switzerland

Summary

(S)-Metolachlor (DUAL MAGNUM®), a potent herbicide of the acylanilide type, has been prepared radiolabelled with carbon-14 in the phenyl ring

The most important herbicide Metolachlor (DUAL®) of NOVARTIS Crop Protection Division is produced since 1978 in volumes of > 20,000 tons per year. In 1997 an enantiomerically enriched form (DUAL MAGNUM®) replaced the racemic mixture leading to a reduction of the environmental load by ca. 40%.

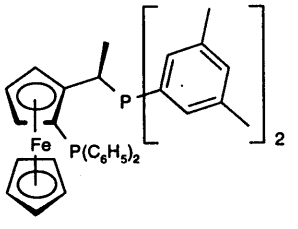
Starting from 2-ethyl-6-methyl[ring-U-^{14}C]aniline **(1)** the synthesis of (S)-[^{14}C]Metolachlor **(4)** was prepared in 3 steps in an overall yield of 70%.



[^{14}C]MEA
1

[^{14}C]S-DUAL
4

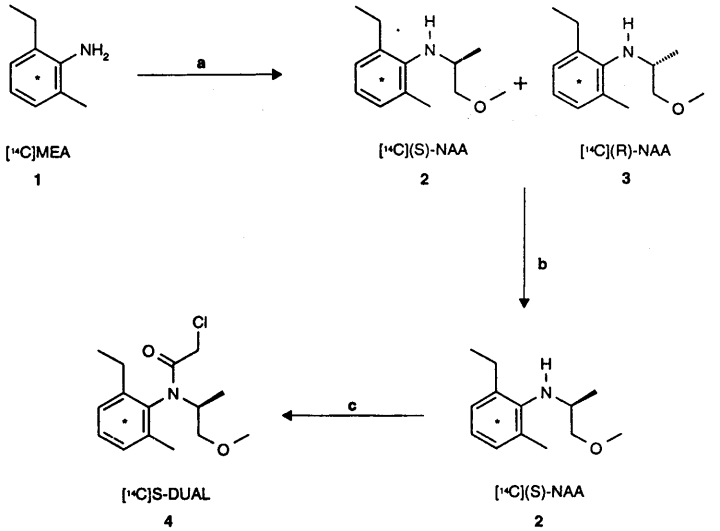
The key step of the radiosynthesis is the enantioselective reductive alkylation of 2-ethyl-6-methyl[ring-U-^{14}C]aniline **(1)** using a novel, highly active iridium ferrocenyl-diphosphin catalyst (PPF-P(3,5-xyl)$_2$ / [Ir(COD)Cl]$_2$).



PPF-P(3,5-xyl)$_2$

The enantioselectivity of this reaction depends strongly on the hydrogen pressure and the temperature used. At about 200 bar the enantiomers **2** and **3** are formed in a ratio of about 90 : 10.
Enantiomers **2** and **3** can be separated by preparative HPLC using a Daicel Chiralcel OD - column. Pure **2** is then reacted with chloroacetyl chloride to give the final compound (S)-[^{14}C](Metolachlor **(4)**.

Scheme I



a : methoxyacetone, catalyst: PPF-P(3,5-xyl)$_2$ / [Ir(COD)Cl]$_2$., tetrabutyl ammonium iodide, methanesulfonic acid, cyclohexane, hydrogen pressure 200 bar, 0 - 25°C

b : separation of the enantiomers **2** and **3** on Daicel Chiralcel OD (Daicel Chemical Industries Ltd.)

c : chloroacetyl chloride, pyridine, dichloromethane, 0 - 25°C

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Preparation of [^{14}C]Ribantoin, a Naturally Occurring Spironucleoside

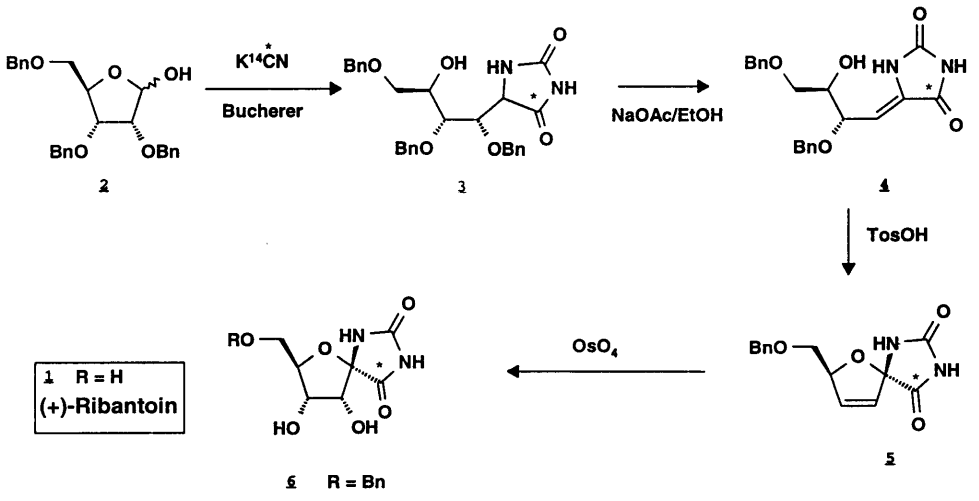
P. Ackermann[1], S. Mirza[2] and P. Thür[1]

[1] Novartis Crop Protection AG, Isotope Laboratory Synthesis, CH-4002 Basel, Switzerland

[2] Novartis Crop Protection AG, Agro Research Computing, CH-4002 Basel, Switzerland

(+)-Hydantocidin (**1**) is a natural spironucleoside isolated from the fermentation broth of *Streptomyces hygroscopicus* SANK 63854 (1); Tü-2474 (2) and A-1491 (3). It exhibits an interesting profile of growth-regulatory and herbicidal activities against monocotyledonous an dicotyledonous annual weeds, with no toxicity to microorganisms, fungi, fish and mice (4). The unique structure of **1** provides the first example of a nucleoside with anomeric spiroconnection between the sugar moiety (D-ribose; therefore named Ribantoin (2)) and the hetercycle (hydantoin,named Hydantocidin (1)). It was shown, that (+)-hydantocidin is the only one of 16 possible diastereoisomers at the four contiguous stereogenic centers to be biologically active. Its intriguing structure and remarkable biological properties have stimulated a considerable amount of synthetic work on the parent compound (5) and its analogues (6). The novel injury symptoms it elicits in plants has prompted a detailed investigation of its mode of action. For these studies radiolabelled material was required. Due to the low fermentation yield we had to develop a synthesis for the preparation of (+)-[^{14}C]hydantocidin, bearing the label in position 9 of the hydantoin ring. Studies of the previous syntheses showed, that the main challenge is the control of the configuration at the anomeric center. This difficulty is compounded by the fact that the isomer bearing nitrogen in the α-anomeric position is thermodynamically more stable than the β-isomer. In our synthetic strategy, tribenzoyloxyribose **2** was used as a chiral building block. As outlined in Scheme 1, *Bucherer-reaction* together with interesting transformations of the formed hydantoins **3** & **4** led in four steps to (+)-[^{14}C]hydantocidin **1**.

With the aid of labelled **1** it was shown, that (+)-hydantocidin is a proherbicide that, after phosphorylation at the 5' position inhibits adenylosuccinate synthetase (AdSS) (7), an enzyme involved in *de novo* purine synthesis.



Scheme 1

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Acknowledgements

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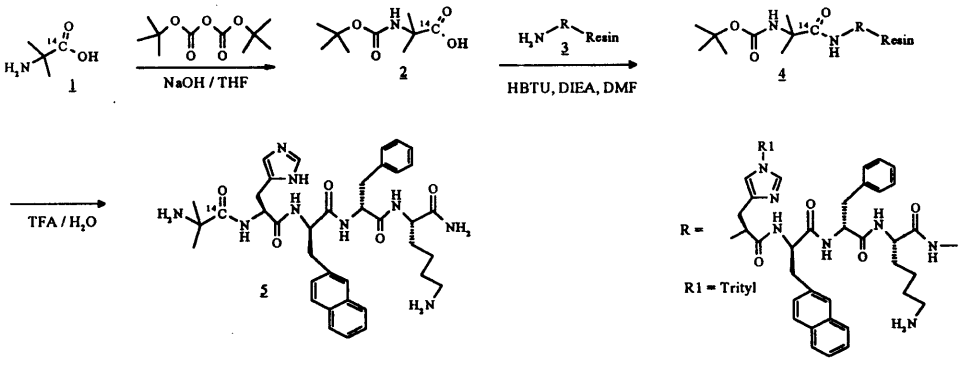
^{14}C-Labelling of Ipamorelin a Growth Hormone Secretagogue

Lars Müller[1], Kjeld Madsen[2] and Christian Foged[1]
Isotope Chemistry[1] and Med.Chem.Research IV[2], Novo Nordisk A/S,
Novo Nordisk Park, DK-2760 Maaloev, Denmark

We have developed a method for the preparation of [^{14}C]α-Aib-His-D-2Nal-D-Phe-Lys-NH_2 ([^{14}C]ipamorelin), from α-2-amino[1-^{14}C]isobutyric acid (1) using solid phase chemistry. To 1 dissolved in water was added sodium hydroxide (aq), THF and di-tert-butyl-pyrocarbonate and the reaction was performed at room temperature. Radio-TLC analysis showed a 90% radiochemical conversion of 1 to 2. HBTU, N-ethyldiisopropyl amine and 3 dissolved in DMF were added to 2, pH was 9-10. The grey suspension was stirred over night at room temperature. The incorporation of radioactivity was followed by centrifugation of the homogeneous mixture. This separated the resin bound [^{14}C]ipamorelin (4) from the free 2 which was dissolved in the supernatant. The precipitate consisted of a mixture of the non-radioactive 3 and 4. Samples were taken from the supernatant and the amount of radioactivity was determined using LSC. Deprotection, using TFA/water (95/5), was followed using the same technique.

Scheme: Preparation of [^{14}C]ipamorelin (5)



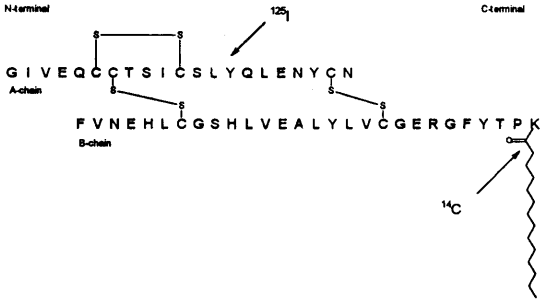
Purification was performed on a C-18 semi-preparative HPLC system using ammonium sulphate/acetonitrile as the HPLC-eluent. Evaporation of the HPLC fractions *in vacuo* resulted in huge amounts of inorganic salts. Instead of evaporation *in vacuo* the pooled HPLC fractions (800 ml) were eluted through a C-18 SepPak thus avoiding salt contamination of the final product. The [^{14}C]ipamorelin was eluted from the SepPak using 0.1% TFA/acetonitrile (30/70, 6 ml) and freeze-dried overnight. The dry material was dissolved in water. The overall radiochemical yield was 31%. The radiochemical purity was >98% and the specific radioactivity for [^{14}C]ipamorelin was 57 mCi/mmol as determined by MS. The radiochemical concentration was 2.3 mCi/ml. Stability studies performed after 8 month storage at -25° C showed only minor radiochemical degradation (less than 1%).

^{125}I- AND ^{14}C- LABELLING OF THE LONG-ACTING INSULIN DERIVATIVE NN304.

Jesper B. Kristensen[a], Lars K. Müller[a], Ulla D. Larsen[a], Louis B. Hansen[b] and Christian Foged[a]

Novo Nordisk A/S, [a]Isotope Chemistry and [b]Chemical Development, Novo Nordisk Park, DK-2760 Måløv, Denmark.

The synthesis of Lys$^{\varepsilon B29}$ [1-^{14}C]-Tetradecanoyl des(B30) human insulin ([^{14}C]-NN304) and [^{125}I]-Tyr^{A14} NN304 used for absorption, distribution, metabolism and excretion (ADME) studies are described.



[^{14}C]-NN304 was synthesised in two steps starting from 1-[^{14}C] myristic acid . The first step being the formation of [1-^{14}C]tetradecanoate N hydroxysuccinimid by reaction of the 1-[^{14}C] myristic acid and N-hydroxy-succinimide in the presence of a dehydrating agent, N,N′-dicyclohexylcarbodiimide. In the second step the [1-^{14}C]tetradecanoate N-hydroxysuccinimid ester and des(B30) human insulin was linked in a mixture of 1-methyl-2-pyrrolidone (NMP) and water using N,N-diisopropylethylamine (DIEA) as the base. A two step purification procedure was performed. The first step was ion-exchange chromatography which removed more than 90% of the impurities. However, to obtain a radiochemical purity >98% a subsequent reverse phase purification on a C-4 column was needed. The overall radiochemical yield was 15% with a radiochemical purity >98%.

NN304 was radio iodinated using the lactoperoxidase/hydrogen peroxide method. The amount of oxidising reagent (hydrogen peroxide) used was equimolar to the amount of ^{125}I. This means that the addition of a reducing reagent to stop the reaction (which could be harmful to the sulphur bridges in insulin) was avoided. The molar amount of NN304 relative to ^{125}I was kept above 100:1 in order to avoid the formation of double labelled NN304 molecules. The Tyr A14 isomer was isolated using a isocratic C18 RP HPLC method. The radiochemical yield was 30% with a radiochemical purity of >98%.

SYNTHESIS OF RADIOLABELLED VERSIONS OF GV150526 A NOVEL N-METHYL-D-ASPARTATE RECEPTOR ANTAGONIST

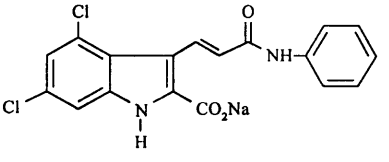
Mario Almi and Simone Guelfi

Glaxo Wellcome S.p.A., Chemical Development Department,
Via Fleming 4, 37135 Verona, Italy
email: sg21148@glaxowellcome.co.uk

Introduction

The pivotal role of the N-methyl-D-aspartate (NMDA) receptor complex in different neurodegenerative pathologies such as ischaemic or haemorragic stroke has been widely recognised [1]. In the last decade glycine has gained considerable interest among different modulators of the NMDA receptor, in view of its key functional role as *co-agonist* of the glutamate in the activation of this receptor. Therefore, in the last few years, the glycine binding site associated with NMDA receptor has been perceived as a target and considerable efforts have been devoted to find new classes of ligands which, acting as glycine antagonists, could be effective neuroprotective agents.

As part of the research for new glycine antagonists, a novel class of indole-2-carboxylates has been investigated [2]. Among these **GV150526 (1)** has shown high receptor selectivity and good *in vivo* activity, therefore carbon-14 and tritium labelled versions were required for drug development studies.

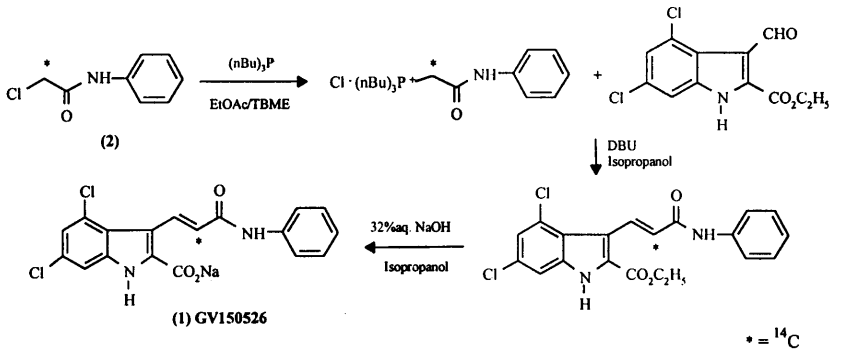


(1) GV150526

Carbon-14 Labelled GV150526

The carbon-14 labelled version of GV150526 **(1)** has been synthesised *via* 2-[*chloroacetyl*-2-^{14}C]chloroacetanilide **(2)** [3], introducing the carbon-14 label at the metabolically stable C-2 position of the side chain (Scheme 1). The final title compound [^{14}C]GV150526 **(1)** was obtained in 55% overall radiochemical yield (99% radiochemical purity by HPLC and TLC).

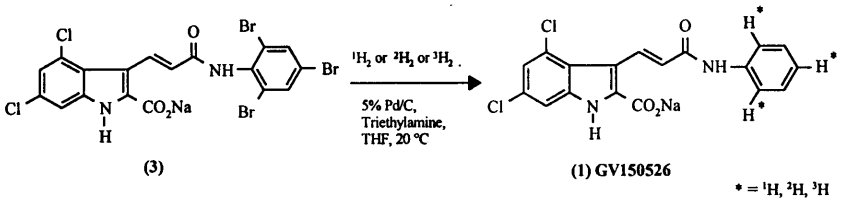
Scheme 1: Synthesis of Carbon-14 Labelled GV150526



Tritium Labelled GV150526

A tritium labelled version of GV150526 (**1**) at high specific activity, for receptor binding studies, has been obtained by selective catalytic dehalogenation [4] of the tribromurated derivative (**3**) (Scheme 2). During the cold study the experimental conditions have been settled in order to minimise dechlorination and over reduction of the double bond. The reaction behaviour was confirmed by employing deuterium gas and HPLC-MS/MS analysis. The procedure was then repeated with tritium gas and proceeded as expected affording [^{3}H]GV150526 (**1**) at 2.59TBq/mmol (70Ci/mmol, radiochemical purity > 98%) after HPLC purification [5].

Scheme 2: Synthesis of Tritium Labelled GV150526



References and notes

[1] a) Meldrum, B.S. *et al., TiPS*, **1990**, *11*, 379. b) Mc Cullogh, J. *Br. J. Clin. Pharmacol.* **1992**, *34*, 106.
[2] Di Fabio, R. *et al., J.Med. Chem.* **1997**, *40*, 841-850.
[3] 2-[*chloroacetyl*-2-^{14}C]chloroacetanilide was purchased from Amersham International plc.
[4] Brundish, D.E. *et al., J. Lab. Comp. Radiopharm.* **1988**, *25(12)*, 1361-1369.
[5] Reaction with tritium gas and purification were performed at Amersham International plc.

New results from applications of radioluminography

Jochen Maas
HMR Deutschland GmbH, DI&A, Lead Optimization, DMPK
Building D 528, D-65926 Frankfurt am Main

The „Imaging Plate" is a new film-like radiation image sensor comprised of specifically designed phoshors that trap and store the radiation energy. The stored energy is stable until scanned with a laser beam, which releases the energy as luminescence.

One of the new applications of this technique is the Whole Body Autoradiography: This method, developed by Ullberg, used X-ray films for the detection of radioactive labelled compounds in the organism. Quantification of radioactivity has always been hampered by the small linear range of X-ray films and the tendious procedures involved in densitometry measurement of the exposed film materials. The newly introduced imaging plates offer a more reliable and a very fast way of quantifying the radioactivity distribution in whole-body sections. Today, this new technique (Radioluminography; RLG) is supposed to substitute conventional quantitative distribution studies, i.e. liquid scintillation counting (LSC) of organs/tissues after dissection of the animals. The results presented here are focused on the method cross-validation of RLG vs. LSC and were part of the concerted effort of the European RLG validation group.

In total, 17 organs and tissues were investigated after administration of 14C-labelled compounds to rats. For intra- and interindividual comparisons, LSC was used as a reference method in three ways: Firstly, quantitative determination of radioactivity after conventional dissection; secondly, using tissue punches taken from whole-body sections which had been previously subjected to RLG and thirdly, preparation of tissue samples from the carcasses which remained after preparation of whole body sections. Blood calibration scales were sectioned together with the animals and used to calculate the organ and tissue concentrations after RLG measurements.

Radioactivity concentrations determined using LSC and RLG showed virtually identical values. The paired concentrations (LSC/RLG) of most of the organs and tissues were highly correlated and the slopes of the regression lines close to 1 both for inter- and for intraindividually obtained data. In a few organs and tissues, however, RLG concentrations seems to be under-estimated (adipose tissue and skin); in some organs and tissues, a relatively high variation of concentration data was found (kidneys, adrenals, lungs, testes).

For the inconsistent results found in some organs and tissues, different explanations are possible: The different sample techniques have to be taken into account: for LSC (conventional dissection), exsanguinated animals were used and the whole organs were homogenized, irrespectively of the heterogeneous intra-organ distribution. Furthermore, RLG concentrations were calculated by using blood standards, assuming a blood - equivalent self-absorption of radioactivity for most organs. In cases where the self-absorption was significantly different from blood, the accuracy of RLG concentration data was reduced (e.g. adipose tissue, skin).

To sum up, the inter-method comparison shows that both techniques, LSC and RLG, showed virtually identical results. With few limitations, RLG is a validated and useful tool for quantitative distribution studies and may substitute LSC. Compared with the LSC and conventional dissectin, results of similar or even higher quality may be expected. Prerequesite are strictly standardized procedures for the RLG.

Experiences with the Micro-imager™

Gisela Backfisch, Boehringer Mannheim GmbH, Analytical Department TR-CAI, Sandhoferstr. 116, 68298 Mannheim, Germany

The Micro-imager™ (Biospace Measures, Zinsser) is a patented detector for radioactivity, which performs real time analysis of tissue sections on a 9x13 mm^2 area at least 30 times faster than a film and 100 times faster than emulsions. It offers a 15 μm spatial resolution and performs a high β detection efficiency leading to a wide dynamic range. It delivers absolute linearity and has a detection efficiency ranging from 60-100 %, depending on both the labelling (^3H or ^{14}C for example) and the noise rejection threshold.

The detection principle is based on the combination of an intensified CCD camera and a thin high resolution scintillator. The energy of an emitted particle, which crosses the scintillator sheet, is partly converted into light. An image intensifier tube multiplicates the signals and the resulting secondary electrons are focused onto a luminescent screen.

This amplified signal is supplying an intensified CCD camera, which locates the resulting output spot and analyses its corresponding light intensity. The successive detected events are accumulated in real time and displayed on a PC-monitor.

This instrument was used in our group to answer very special questions, with the demand for a high resolution. For example: we need to detect ^3H and ^{14}C in the same section of the rat kidney. We found that both compounds were concentrated in the pelvis also in the same area, which suggest a very similar elimination rate.

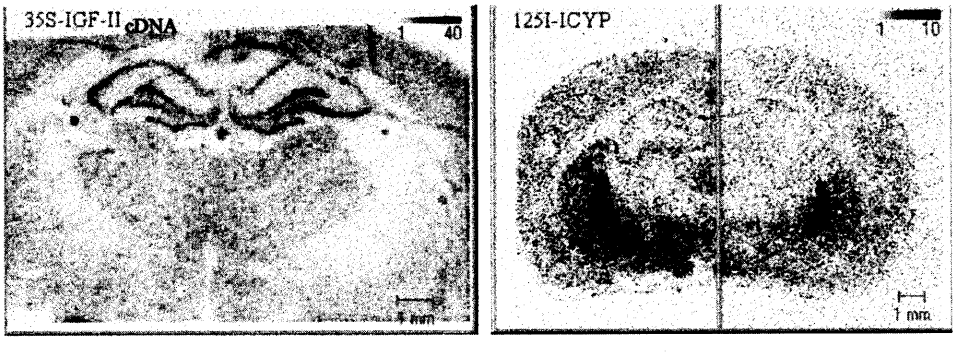
Another question was the distribution of the radioactivity of ^{14}C in the bone. With the high resolution of the Micro-Imager we could locate the radioactivity only in the growing regions of the joint of the rat femur.

The Micro-Imager is an instrument which allows to give a closer look at tissues. It is the link between normal autoradiography of whole animals or organs and the real micro-autoradiography of on a cellular approach.

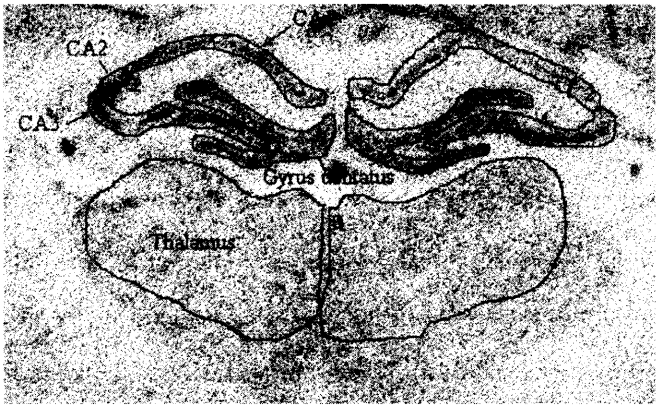
Lateralization of molecular expression patterns in the brain of rats analyzed by microimager autoradiography.

A. Zimmermann, S. Milanovic, N. Schertel and F. Henn
Biochemisches Labor, Zentralinstitut für Seelische Gesundheit, J5, 68159 Mannheim, Germany.

Studies on human cognitive functions have suggested that the left hemisphere serves well routinized representations and strategies and the right one novel cognitive situations (1). We have been interested in addressing the topic of lateralized or bilateral brain activity at the molecular level in the context of research on depression analyzing learning behavior under stress. Here, we report on the use of a new autoradiography system, Microimager Autoradiography and Beta-Vision Software (BIOSPACE) for autoradiographic data acquisition and analysis. The expression of the β_2 adrenergic receptor and IGF-II gene was analyzed on sections of rat brain using ^{125}I-Iodocyanopindolol (^{125}I-ICYP) to label β_2 adrenergic receptors and ^{35}S IGF-II cDNA as a probe to detect IGF-II mRNA. The high sensitivity of radiation detection and excellent topographical resolution allowed for the detailed visualization of lateralization in the expression of the IGF-II gene and β_2 adrenergic receptors in various brain regions. IGF-II gene expression as well as β_2 adrenergic receptor binding were found to be clearly left lateralized.

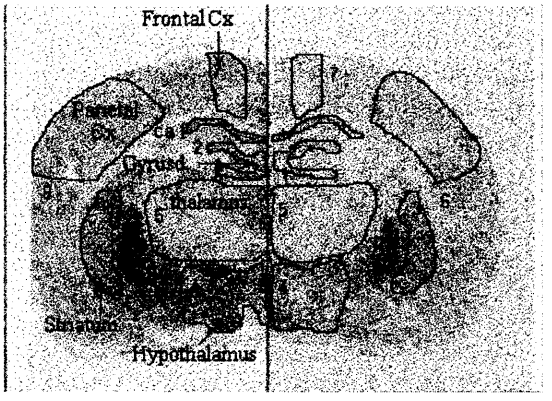


In addition, the system proved to be highly suited for obtaining quantitative data on the lateralization of gene expression and receptor binding, e.g., in the hippocampus, IGF-II gene expression was by about 200% higher in the left dentate gyrus, by about 100% higher in the left CA2 area, and by about 20% higher in the left CA1 area compared to the right hemisphere, while no lateralization was seen in CA3 on the sections analyzed. Neither was there significant thalamic lateralization of IGF-II gene expression.



		IGF-II mRNA expression/ SD brain				
		Count	dpm	cp/mm^2	Surface/ mm^2	% difference (Left/Right)
Gyrus dentatus	Left	21228	22,1	18,69	1,18	**239.8**
	Right	5948	6,19	5,5	1,13	
CA3	Left	14383	14,98	19,32	0,78	-8.3
	Right	18792	19,57	21,06	0,93	
CA2	Left	2464	2,57	24,02	0,11	**97.5**
	Right	1698	1,77	12,16	0,15	
CA1	Left	12645	13,17	16,38	0,8	**23.4**
	Right	14898	15,51	13,27	1,17	

In ^{125}I-Iodocyanopindolol (^{125}I-ICYP) binding to β_2 adrenergic receptors, left-right side differences were likewise observed, and were in the range of 30% in the dentate gyrus and CA1 area, 25% in thalamus, about 40% in striatum and hypothalamus, 50% in frontal cortex and 10% in parietal cortex. First results from these molecular imaging studies suggest that differences in lateralization may underlie behavioral characteristics of depression seen in the animal model.



		Beta 2 adrenergic receptor distribution/ SD brain				
		Count	dpm	cp/mm^2	Surface/ mm^2	% difference (Left/Right)
Gyrus dentatus	Left	1269	10.56	7.24	1.46	**27.0**
	Right	848	7,06	5,7	1,24	
CA1	Left	846	7,04	8,19	0,86	**29.4**
	Right	542	4,51	6,33	0,71	
Striatum	Left	2979	24,79	12,16	2,04	**32.5**
	Right	2539	21,13	9,18	2,3	
Hypothalamus	Left	8014	66,68	13,32	5,01	**35.1**
	Right	6182	51,44	9,86	5,22	
Thalamus	Left	8080	67,23	7,21	9,32	**24.0**
	Right	4972	41,37	5,81	7,12	
ParCx	Left	1885	15,68	8,21	1,91	12.0
	Right	5935	18,02	7,33	2,46	
FrontalCx	Left	2597	21,61	7,26	2,97	**53.8**
	Right	1542	12,83	4,72	2,72	

ON–LINE MONITORING OF α–PARTICLES IN THE PRESENCE OF β–EMITTERS BY SOLID SCINTILLATION COUNTING

U. Wenzel*, W. Ullrich*, D. Freystadt**
*Forschungszentrum Jülich GmbH, Institut für Sicherheitsforschung und Reaktortechnik,
D-52425 Jülich, ** EG&G Berthold GmbH & Co. KG, D-75323 Bad Wildbad

The LB 508 Radioactivity Monitor of EG&G Berthold is well known for the online detection of radioactivity in HPLC. For detecting α-emitting nuclides in the presence of β-emitters two detection methods are available. The *heterogeneous method* is characterized by using a tube which is filled with a special solid scintillator grain. In contrast to this principle the *homogeneous method* with the admixture of liquid scintillator results in better efficiencies especially for low range radiation. These two methods show several disadvantages. The solid scintillator causes a build up of backpressure and may itself react as a chromatographic column with memory effects. The cocktail is often responsible for quenching effects and may result in waste disposal problems due to its toxic properties. None of these disadvantages are really significant in HPLC. The used solvents have good elution properties and analytical HPLC produces minimal waste. On the other hand the disadvantages limit the use of the LB 508 to monitor HPLC separations.

Within our R&D program relating to the treatment of actinide bearing wastes we tried to enlarge the field of applications for the LB 508 to the online control of processes with technical scale. For that we established the following conditions: 1) low pressure (< 2 bar) operation at high flows, 2) no memory effects, 3) no additional wastes. We achieved these conditions by coating the inner surface of the heat resistant translucent plastic tubes with meltable plastic scintillator MELTILEX™. Tubes with an inner diameter ϕ_i > 3mm were coated by vortexing liquid MELTILEX™ through the tubes with a hot air stream. Tubes with ϕ_i < 3 mm were completely filled with molten scintillator and then blown out with hot air leaving a thin adhering scintillator film at the tube wall.

The detectors show a good efficiency ε for high and medium energetic β–particles (~ 70 % for Y-90 and Rh-106, ~ 25 % for Sr-90, Cs-134 and Cs-137) independent of the tube geometry. The efficiency for low energetic β– and for α–particles was low, but increased with decreasing the diameter ϕ_i ($\varepsilon_{Cm\text{-}244}$ = 3 % at ϕ_i = 0.28 cm; $\varepsilon_{Cm\text{-}244}$ = 11.5 % at ϕ_i = 0.04 cm.). We also observed that β–particles interacted with the scintillator producing mainly low energy pulses, while α–particles generate a significant amount of high energy pulses (see Figure 1). For that reason we established the concept of the Multi Loop Bundle Detectors for monitoring α–bearing solutions. We coated 1/16 inch tubes (ϕ_i = 0.05cm) and fitted together seven identical loops by sticking their ends into 8 mm supporting hulls. The ends were then connected to the bulkhead unions of the LB 508 detector cover plate. With this experimental set-up we could reduce the pressure in the detector loop to 1.9 bar at a flow of 60 ml/min. Table 1 presents the characteristics of the bundle detector. The detector shows practically no memory effect. Pumping a 0.25 M HNO_3-solution containing Cm-244 through the detector loop the maximum signal was reached after 2 loop volumes. Scrubbing the detector we reached background activity after 4 loop volumes due to vortexing effects between feed and scrub solution (0.25 M HNO_3). Table 2 summarizes the efficiencies and detection limits for α–emitters at

various energy windows. By setting energy windows a decrease of the efficiency is observed. The corresponding detection limits however improve due to the lower background signal in that window. We used the different interaction of α– and β–particles for discrimination demonstrated in Figure 2. We separated a mixture of Sr-90/Y-90/Cm-244 with 2000 Bq/ml for each nuclide using a front chromatography. We measured online the specific activity λ in the column eluate at three energy windows. In the first 90 channels, 93 % of the pulses were generated by Sr-90 (30%) and Y-90 (63 %). Setting the window to 301 -999 channels, 87 % of the pulse yield came from the α–emitter Cm-244 (Sr-90 ≡ 3 %, Y-90 ≡ 10 %)

The detector is highly selective for α–emitters. In fission product solutions with a β–activity of $2x10^9$ Bq/ml, the detector was able to measure a specific Cm-244 activity of 25000 Bq/ml corresponding to the amount of 0.25 % Cm-244 in such solutions.

Table 1

WUW-ML Bundle Detector

number of loops	loop length	ϕ_i of loop	cell volume	scint. surface
	[cm]	[cm]	[cm^3]	[cm^2]
7	50	0.04	0.46	45.1

Table 2

Efficiencies and Detection Limits

energy range [channels]	2 999	101 999	201 999	301 999	401 999
background [cps]	2	0.32	0.083	0.025	0.017
efficiency [%]	11.5	6.1	3.54	2.26	1.02
detection limit [Bq/ml]	8.1	6.1	5.3	4.6	8.3

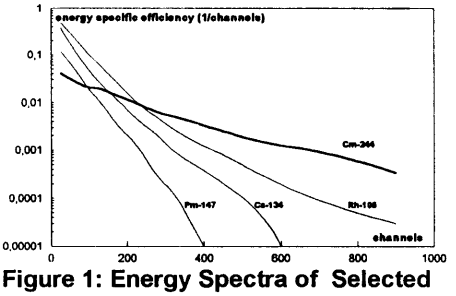


Figure 1: Energy Spectra of Selected Nuclides

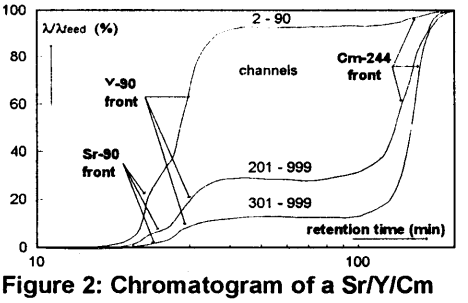


Figure 2: Chromatogram of a Sr/Y/Cm Separation

Radioactivity Detection in Line with Mass Spectrometry

Günter Dietzel, Dipl. Ing.,
Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany

The combination of radio-chromatography with mass spectrometry is getting more and more applied.

Conventional radioactivity flow detectors for HPLC are not suitable for the combination with mass spectrometers.

In particular radioactivity flow detectors with admixture of liquid scintillator require a stream splitter. One part of the HPLC eluate is diverted to the injector of the mass spectrometer. The other part of the HPLC eluate is admixed with liquid scintillator and flows through the radioactivity detector.

Many researchers have tried this configuration and found, that it is very difficult or almost impossible to obtain reliable splitting. The backpressure at the input of the mass spectrometry injector is much higher compared to the liquid scintillator admixture system.

Solid scintillators with a particle size of 5 - 10 µm have a detection efficiency of about 90 % for C 14 and 20 % for H 3.

Solid scintillators are available from various materials and undisolvable for almost all applications of eluate mixtures and gradient profiles.

Glass scintillators can be coated with silicon groups, which avoid memory effect of the labeled compound on the scintillator bead.

Solid scintillator cells for combinations with mass spectrometers can be manufactured with volumes from 5 µl to 100 µl. Dead volumes can be reduced to a few µl.

Application tests with various cell volumes demonstrate the effect of peak broadening versus detection sensitivity of a flow cell.

Several results of RI/MS-chromatograms, obtained by several researchers, are demonstrating the usefulness and quality of the method.

Sensitivity and reproducability is demonstrated at 10 dpm/peak-^{14}C.

Limit of detection is about 20 dpm/peak-^{14}C.