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Some Selected Abstracts Relevant for Radiosynthesis and Applications of Labelled Compounds

Edited by

Rolf Voges

Drug Metabolism and Pharmacokinetics, Isotope Section,
Novartis Pharma AG, CH-4002 Basel, Switzerland

Ulrich Pleiss

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

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TETRAMETHYLAMMONIUM TRI-ACETOXYBOROTRITIDE, A REAGENT FOR HYDROXY-DIRECTED KETONE REDUCTION

Hendrik Andres

Preclinical Safety-Drug Metabolism & Pharmacokinetics, Isotope Section
Novartis Pharma AG, CH-4002 Basel
Hendrik.Andres@Pharma.Novartis.com

Discodermolide (3, T=H) is a novel cytotoxic, polyketide natural product that was isolated from the marine sponge *Discodermia dissoluta*. Its synthesis[1] involves a diastereoselective ketone reduction at the end of the synthetic pathway. The reagent of choice is tetramethylammonium tri-acetoxyborohydride[2] because the borohydride coordinates with the substrate by ligand exchange and the tetramethylammonium cation does not chelate with the oxygen atoms in ß-position to the carbonyl group to be reduced. Thus the reagent is able to attack the precursor 1 from the less hindered side yielding the anti-diol which spontaneously cyclises with simultaneous elimination of MeONHMe. The resulting mixture (intermediate 2 and the corresponding uncyclised secondary alcohol, see **Scheme 1**, unlabelled isotopomers) gives discodermolide after deprotection in a diastereomeric purity of >97%. The total synthesis from commercially available building blocks involves at least 30 chemical steps. The carbon-14 synthesis required very expensive starting materials, both for labelled and unlabelled precursors. Alternatively, labelling with tritium promised to be short, less expensive and would provide a higher specific activity. The labelling reagent however, as well as its sodium and potassium analogs, were so far unknown in tritium chemistry.

Lithium tri-acetoxyborotritide can easily be formed from tri-acetoxyborane and lithium tritide in dimethoxyethane. Attempts to produce the target reagent by reaction with tetramethylammonium salts containing anions, which might be able to remove lithium ions by precipitation of insoluble salts, failed under various conditions and in various solvents. However, it was possible to convert the corresponding sodium tri-acetoxyborohydride to its Me_4N-analog by simply dissolving a mixture of Me_4NCl and $Na(OAc)_3BH$ in acetonitrile/acetic acid 1:1 at 0°C. NaCl precipitated nearly quantitatively and the supernatant was able to effect the desired reduction diastereoselectively.

The tritiated reagent was obtained in a three-step process:

1. preparation of $NaBT_4$ by high temperature exchange reaction

$$NaBH_4 \xrightarrow[450°C]{T_2} NaBT_4$$

2. preparation of $Na(OAc)_3BT$ by reaction with 3.5 equivalents of acetic acid in benzene at room temperature

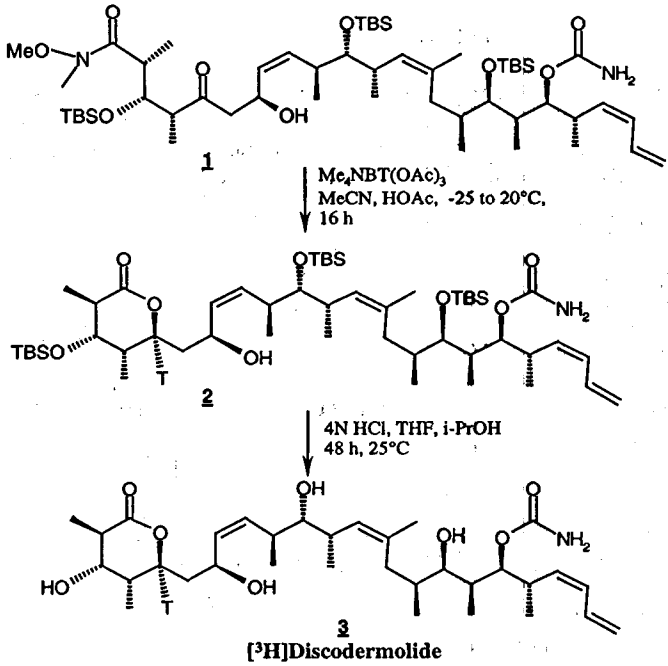
$$NaBT_4 + 3\ AcOH \longrightarrow Na(OAc)_3BT + 3\ HT$$

3. exchange of the counterion by reaction with Me_4NCl in acetonitrile/acetic acid

$$Na(OAc)_3BT + Me_4NCl \xrightarrow{AcCN/HOAc} Me_4NB(OAc)_3T$$

4. For the reduction of the precursor of Discodermolide an excess of the reagent was used because of the scarcity of the precursor

Scheme 1: Synthesis of [^{3}H]Discodermolide



[^{3}H]Discodermolide

Deprotection of intermediate **2** finally furnished [^{3}H]Discodermolide at a specific activity of 252 GBq/mmol.

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SYNTHESIS OF TRITIUM-LABELED NUCLEOSIDES WITH ANTI-HIV AND ANTICANCER ACTIVITY

G.V. Sidorov*, Yu.B. Zverkov*, N.F. Myasoedov*, A.R. Cimpoia* and A.B. Susan***

ASUSAN@kc.rr.com

* Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov Sq. 2, 123182, Moscow, Russia,
** BioChem Pharma Inc., 275 Armand-Frappier Blvd., Laval, Quebec, Canada, H7V 4A7
*** International Isotope Clearing House Inc., 9110 Lee Blvd., Leawood KS 66206, USA

INTRODUCTION

The development of new antiretroviral drugs active against multidrug resistant HIV strains prompted the synthesis of tritium labeled nucleosides. These compounds (I-V) were synthetic cytidine and guanosine analogues which were designed to cause DNA chain termination due to the lack of 3'-hydroxyl group.

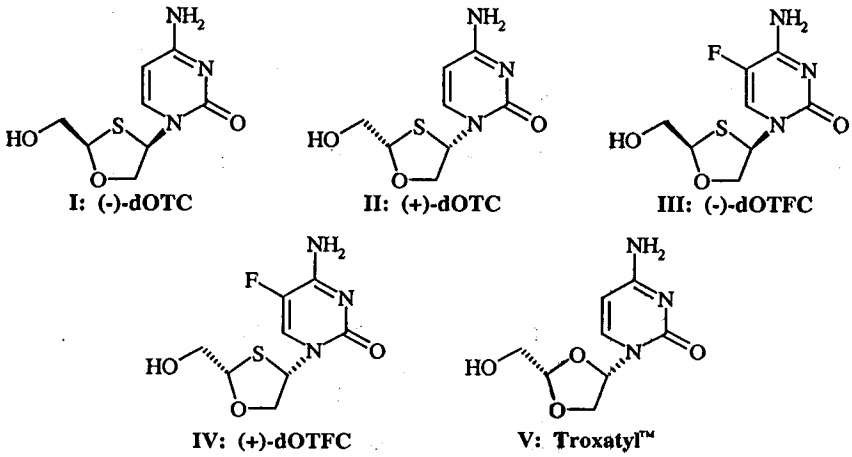

Figure 1: Compounds I->V selected for tritium labelling

I: (-)-dOTC i.e.(-)2'-Deoxy-3'-oxa-4'-thio-cytidine, **II: (+)-dOTC**, i.e. 2'-Deoxy-3'-oxa-4'-thio-cytidine, **III: (-)-dOTFC** i.e. 2'-Deoxy-3'-oxa-4'-thio-5-fluorocytidine, **IV: (+)-dOTFC** i.e. 2'-Deoxy-3'-oxa-4'-thio-5-fluorocytidine, and **V: TROXATYL™** i.e. **2'-Deoxy-3-oxacytidine.**

TROXATYL™ (V) is a L-nucleoside with potent in vitro and in vivo antitumor activity. This cytidine analog is a DNA polymerase inhibitor and a complete DNA chain terminator.

Compounds **I - IV** were labeled using the solid state catalytic hydrogen exchange reaction with tritium gas [1]. The catalytic halogen exchange reaction of a corresponding 5-iodo-substituted derivative was used for the synthesis of tritium-labelled **TROXATYL™** (V). No racemization of either chiral centre occurred during the introduction of tritium in all studied compounds and the optical purity of labelled compounds was equal to optical purity of the corresponding non-labelled compounds.

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TRITIDE REDUCTION OF NUCLEOSIDES AND CARBOHYDRATES

Manouchehr Saljoughian*, Hiromi Morimoto, Philip G. Williams, Chit Than

The National Tritium Labelling Facility and Physical Biosciences Division, Lawrence Berkeley National Laboratory, University of California, One Cyclotron Road, Berkeley, CA 94720, USA.
e-mail: MSaljoughian@lbl.gov

The utility of several H-2 and H-3 labeled hydrides in the regio- and stereoselective deuterium and tritium labeling of D-glucose and of several nucleosides is reported. The C-1 position of D-glucose was labelled by tritide reduction of a glucono-lactone precursor. Deuteride/Tritide-reagents were also employed for the labelling of the 2', 3' or 5',5"-positions of the ribose and deoxyribose moiety in adenosine, uridine and thymidine. In each case, a different synthetic pathway was required to make the desired precursor and a different deuteride/tritide was utilized for the reduction to produce the respective specifically labeled product.

Examples of the experiments are listed below exploring the scope and limitations of the reagents and methods applied.

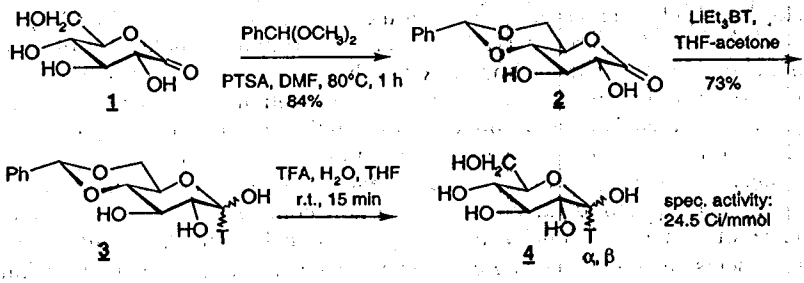
1. The synthesis of high specific activity D-[1-^{3}H]glucose **4** started from 4,6-*O*-benzylidene-D-glucono-1,5-lactone **2** (Scheme1). It was conveniently prepared by benzylidation of D-glucono-1,5-lactone **1** with benzaldehyde dimethyl acetal under acid catalyzed conditions [1]. Lithium triethylborotitide (supertritide, $\mathbf{LiEt_3B^3H}$) reduction of the benzylidene lactone at –78°C furnished 4,6-*O*-benzylidene-D-[1-^{3}H]glucose **3** with one atom of tritium at the C-1 position. Deprotection of the benzylidene group gave pure tritiated D-glucose **4** in high chemical yield and high specific radioactivity. ^{1}H- and ^{3}H-NMR analyses showed the expected α-C1 and β-C1 anomers of tritiated glucose.

2. 2'-Deoxy-[2'*R*-^{3}H]adenosine has been synthesized by selective deoxygenation. The hydroxyl group at the 2'-position of the ribose sugar was replaced *via* free radical deoxygenation of the adenosine-2'-*O*-phenoxythiocarbonate ester [2,3] using high specific activity n-tributyltin tritide ($\mathbf{n\text{-}Bu_3Sn^3H}$). This experiment illustrates the first chemical synthesis by 2'-deoxygenation of a nucleoside (adenosine) with the incorporation of a tritium atom at the 2'-position of the sugar to generate 2'-deoxy[2'*R*-^{3}H]adenosine at very high specific activity (20 Ci/mmol).

3. The 3'-position may be labeled as follows: Selective 2',5'-*O*-disilylation of adenosine leaves a free hydroxyl group at the 3'-position. CrO_3 oxidation of the 3'-hydroxyl to give the respective 3'-ketonucleoside and removal of the 5'-silyl group, followed by sodium triacetoxyborodeuteride ($\mathbf{Na(OAc)_3B^2H}$) reduction of the 3'-ketone will result in the corresponding secondary [3'-^{2}H]alcohol with the desired stereochemistry [4]. The small-scale, non-radioactive synthesis of the nucleoside is underway and the technique will be applied to tritium labeling.

4. [5',5"-$^2H_2$]Nucleosides have been obtained in very high chemical yield with high deuterium incorporation. Sodium borodeuteride (NaB^2H_4) reduction of 2',3'-isopropylideneuridine-5'-*tert*-butyl carboxylate in dry ethanol and lithium borodeuteride (LiB^2H_4) reduction of 3'-TBDMS-*O*-thymidine-5'-*tert*-butyl-carboxylate in dry THF gave the corresponding 5',5"-labeled products. In both cases the ester functionality on the 5'-carbon of the nucleoside precursors was prepared [5] by oxidation of the 5'-primary alcohol of the ribose and deoxyribose using a complex of CrO_3/pyridine/acetic anhydride/*tert*-butanol.

Scheme 1: Synthesis of High Specific Activity D-[1-^{3}H]Glucose



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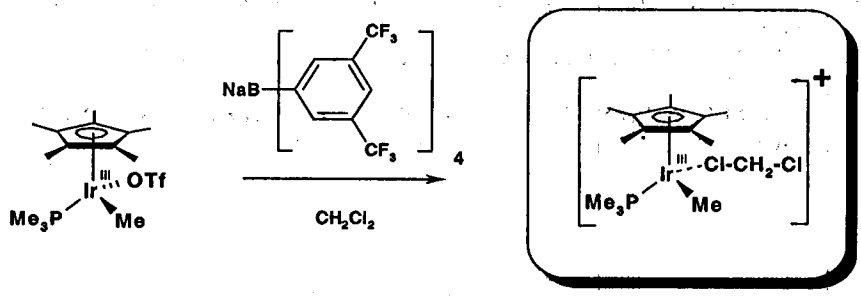
A NEW HOMOGENEOUS COMPLEX FOR H/D/T EXCHANGE LABELLING

R Salter[1], T Moenius[1], P Ackermann[2], M Studer[3], A Morgan[4], M Chappelle[4]

1. Novartis Pharma AG, Preclinical Safety DMPK-IL, 2. Syngenta AG, 3. Solvias AG, 4. Amersham Pharmacia Biotech
e-mail: rhys.salter@pharma.novartis.com

Catalytic H/D/T exchange is a fast, non-synthetic route to isotopomers for drug development and basic research. State of the art catalysts eg. $[Ir(cod)(PCy)_3(py)]PF_6$ allow exchange of Hydrogen for Deuterium or Tritium in molecules of simple to medium complexity[1]. The method is extremely attractive since not only does it give high regioselectivity but also high isotopic incorporation. A major drawback to the approach is that catalysts more often than not fail to label more complex molecules (ie. the real drug substances we are interested in) and except in some specific cases, label only aromatic positions. Some recent work describes a high throughput screening technique to identify a number of new complexes which work well in the presence of some traditionally inhibitory functional groups[2]. While significant improvements in tolerability were achieved using this approach, thus making the technique more applicable to complex substances compared with the state of the art, reliable labelling at alkyl positions remains elusive; as does the possibility to label at positions distal from or more ambitiously in the absence of any coordinating group, which would otherwise be necessary for such catalytic reactions.

The following presentation describes our preliminary results which attempt to overcome these problems. We report the application of the cationic derivative $[Cp^*Ir(PMe_3)(Me)(OTf)]B(Ar_F)_4$ **1** of the Bergman complex[3], to H/D exchange labelling. Complex **1** can be prepared conveniently and rapidly *in situ* (Scheme 1) by addition of an equivalent of sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate in dichloromethane at room temperature.

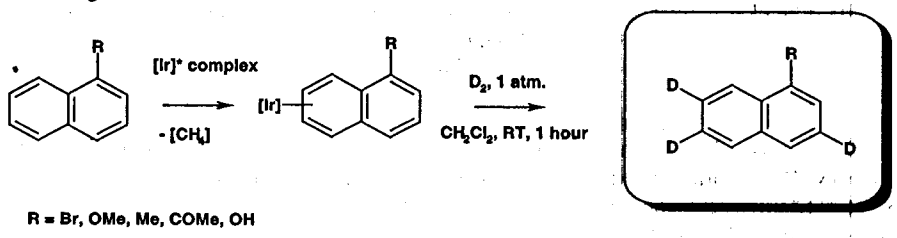


Bergman complex **1**

Scheme 1

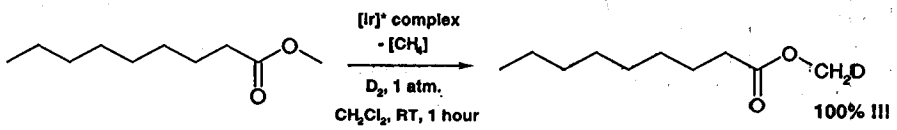
Complex **1** undergoes rapid oxidative addition with a number of substrates bearing no formal coordinating groups in either alkyl or aryl positions[4]. Reductive elimination and concomitant loss of methane affords the corresponding alkylated or arylated derivatives (evidenced by ^{1}H NMR spectroscopy). Subsequent exposure to one atmosphere of deuterium gas at room temperature causes rapid cleavage of the complex and the substrate to furnish the labelled products (schemes 2 and 3). In addition to simple molecules, we examined the outcome of labelling in the presence of a number of different functional groups in order to examine the versatility of this method.

Labelling in aromatics.



Scheme 2

Labelling at alkyl.



Scheme 3

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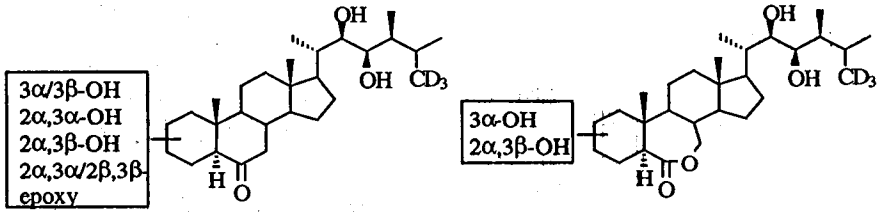
SYNTHESIS OF [26-$^2H_3$]BRASSINOSTEROIDS

Olga V. Konstantinova[a], Vladimir A. Khripach*[a], Vladimir N. Zhabinskii[a], Andrey P. Antonchick[a], Bernd Schneider[b]

[a]Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Kuprevich str., 5/2, 220141 Minsk, Belarus
[b]Max-Planck-Institute for Chemical Ecology, Carl-Zeiss-Promenade 10, D-07745 Jena, Germany
schneider@ice.mpg.de

A number of deuterium-labeled brassinosteroids including [26-$^2H_3$]typhasterol, 2-deoxy[26-$^2H_3$]brassinolide, [26-$^2H_3$]secasterone, 2,3-[26-$^2H_3$]episecasterone, 3-[26-$^2H_3$]epicastasterone, and 3-[26-$^2H_3$]-epibrassinolide has been prepared to study brassinosteroid biosynthesis in plants.



The synthetic route to target compounds included addition of lithium methyl acetylide to C_{22}-aldehyde. The obtained 22α- and 22β-hydroxy isomers were converted into 23*Z*- and 23*E*-allylic alcohols by hydrogenation over Lindlar catalyst and reduction with sodium in liquid ammonia, respectively. Formation of 22,23-double bond and 24*S* center was achieved by Claisen rearrangement of both allylic alcohols. Introduction of deuterium has been done by reduction of the corresponding intermediates by lithium aluminium deuteride.

THE SPECIFIC ACTIVITY DETERMINATION AND CONFIRMATION OF IDENTITY OF A RADIOLABELLED PEPTIDE USING TANDEM MASS SPECTROMETRY

A. T. Rees and A. Colebrook

Nycomed Amersham plc, Cardiff Laboratories, Forest Farm, Whitchurch, Cardiff, Wales CF14 7YT
e.mail: tony.rees@apbiotech.com

Abstract:

Nycomed Amersham, in support of opiate receptor research, manufactures [leucyl-^{3}H]-Nociceptin. It is prepared by the tritiation of [dehydro-Leu]Nociceptin using tritium gas over a palladium catalyst:

Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-[dehydro Leu]-Ala-Asn-Gln

T_2-gas/Pd
$\longrightarrow$ Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-[^{3}H]Leu-Ala-Asn-Gln
DMF, 1hr, r.t.

Specific activity[1,2] of a radiolabelled compound and specificity of the labelled position is of considerable importance to the research scientist. Until now technology has only allowed us to establish the amount of radioactivity associated with the molecular ion and provided no real structural confirmation or indication of the site of labelling. Utilising the power of tandem mass spectrometry we can now quantify and demonstrate the position of the radiolabel within the structure.

The chosen example, [leucyl-^{3}H]Nociceptin, has sites associated with the phenylalanine moieties susceptible to non-specific exchange. The preferred way to demonstrate that this has not occurred is to sequence[3] the peptide and carefully examine the isotope patterns of the isolated residues for evidence of labelling.

Unfortunately not all peptides give satisfactory MS-MS spectra. In this instance they require digestion with an enzyme and chromatographic separation of the resulting fragments prior to MS-MS analysis. This work describes the use of on-line liquid chromatography electrospray. tandem mass spectrometry to achieve this (LC-ESI-MS-MS).

Experimental:

Nociceptin was selectively cleaved using trypsin and Lys-C (Sigma). The fragments were then analysed by HPLC-ESI-MS-MS using a Q-ToF-2™ mass spectrometer (Micromass®, Wythenshawe, UK) linked to a Waters alliance™ HT 2790 separations module with photodiode array detection. The chromatographic conditions were 0 to 100% solvent B over 10 minutes at 300μl/min where solvent A was 0.1% trifluoroacetic

acid(aq) and solvent B acetonitrile containing 0.1% trifluoroacetic acid on a Hypersil® 3μ C18 BDS 50 x 2.0mm i.d. column purchased from phenomenex®. All the solvents were HPLC grade with the exception of the trifluoroacetic acid which was spectroscopy grade purchased from Fluka. The resulting data was interrogated using MassLynx NT version 3.5.

Results:

SEQUENCE **FGGFTGARKSARK(^{3}H)-LANQ-OH**

Enzyme	Fragment	Mass (MH+)	Specific Activity(mCi/mMol)
Endoproteinase Lys - C	FGGFTGARK-OH	940-948	3
	SARK-OH	461	0
	[^{3}H]LANQ-OH	445-461	158
TRYPSIN	FGGFTGAR-OH	812-820	4
	KSAR-OH	461	0
	K[^{3}H]LANQ-OH	589-589	160
NONE	Parent Peptide	1809-1825	165

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Different Carbon-14 Labelling Syntheses of Vardenafil

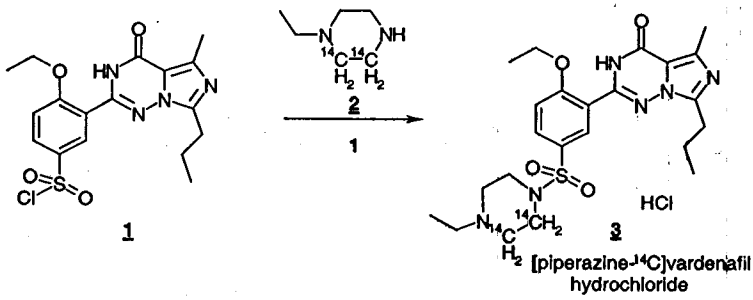
Dietrich Seidel

Bayer AG, PH-PD P Drug Metabolism & Isotope Chemistry
dietrich.seidel.ds@bayer-ag.de

Vardenafil hydrochloride is a new orally active, selective phosphodiesterase type V (PDE V) inhibitor currently under development for the indication male erectile dysfunction [1]. For pharmacokinetics and drug metabolism studies, the compound with a C-14 radiolabel in a metabolically stable position was therefore required.

The first synthesis was performed as shown in **scheme 1**. The label was introduced by reaction of the respective arylsulfonyl chloride **1** with N-ethyl-[2,3-^{14}C]piperazine **2**. The product isolated was treated with hydrochloric acid and purified by recrystallization from ethanol to give the desired [piperazine-^{14}C]-vardenafil hydrochloride **3** in 73% radiochemical yield (8.1 GBq, 2.268 g).

Scheme 1: Synthesis of [piperazine-^{14}C]vardenafil hydrochloride

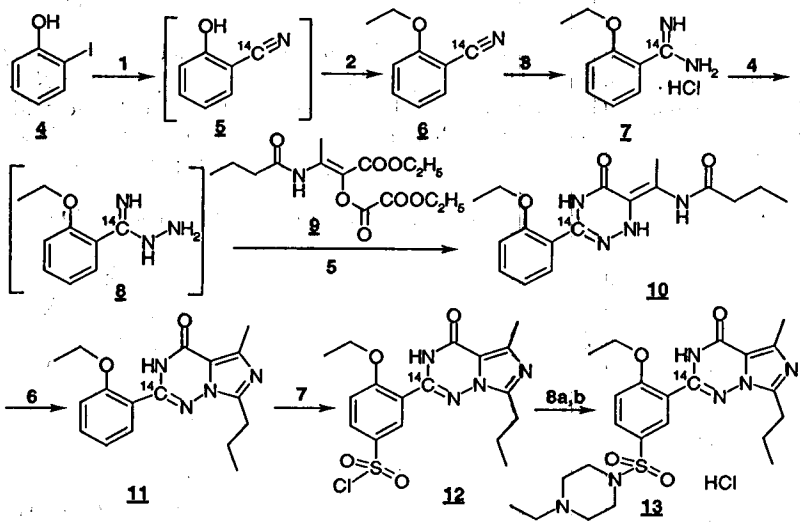


reaction conditions: 1a. 2, $EtiPr_2N$, CH_2Cl_2; r.t., 17 h **1b.** HCl, EtOH,73% **(1a,b; 8.1 GBq)**

Studies later revealed that the label was in a metabolically unstable position and therefore we embarked on an alternative route, which placed the label in the more robust triazine moiety, **scheme 2**. Starting from 2-iodophenol **4** the label was introduced by nucleophilic displacement of iodine by treatment with Cu^{14}CN. The resulting 2-hydroxybenzo[^{14}C]nitrile **5** was reacted with ethyl iodide and potassium carbonate to furnish the aryl ethyl ether **6**. Due to the high amount of starting material (approximately 67 GBq) both reaction steps were performed in three separate runs. After purification on silicagel, **6** was converted to amidine **7** in good yield by reaction with $Al(CH_3)_3$ and ammonium chloride. Hydrazinolysis of **7** gave the corresponding amidrazone **8**, which in the absence of further purification, was reacted with the enolester **9** [2] to give the cyclized triazinone **10**. In order to ensure satisfactory yields in subsequent steps this material was purified by semipreparative chromatography on silica gel. Treatment with $POCl_3$ afforded **11** which on reaction with chorosulfonic acid under mild conditions gave excellent quality and

quantitative yield of **12**. Reaction of **12** with N-ethylpiperazine furnished the free base [triazinone-^{14}C]vardenafil. Finally, the corresponding hydrochloride was precipitated by addition of hydrochloric acid to a solution of the free base in acetone. The product was isolated in excellent yield and required no further purification. The radiosynthesis led to a total radioactivity of 10.7 GBq corresponding to an overall yield of 20% taking an aliquotation in the last but one reaction step into consideration.

Scheme 2: Synthesis of [triazinone-^{14}C]vardenafil hydrochloride



reaction conditions: 1. $K^{14}CN$ (67 GBq), CuI, NMP; 180°C, 5 h **2.** C_2H_5I, K_2CO_3, acetone; reflux, 1.5 h (57 GBq, 85% over two steps) **3.** $Al(CH_3)_3$, NH_4Cl, toluene; reflux 9 h **4.** $N_2H_2 \cdot H_2O$, ethanol, 0°C **5.** **9**, ethanol; reflux 5 h; chromatographic purification on silica gel, 25 GBq, 44% over three steps **6.** $POCl_3$, CH_2Cl_2; reflux, 2 h, 17.9 GBq, 72% **7.** $ClSO_3H$, CH_2Cl_2; 0°C, 1 h; r.t., 16 h, 100% referred to 14.3 GBq of **VII** **8a.** N-ethylpiperazine, $EtiPr_2N$, CH_2Cl_2; 16 h, r.t. **8b.** acetone, HCl (1 equivalent), 10.7 GBq,

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CARBON-14 LABELLING OF β-AMINO ACIDS AND β-PEPTIDES

M. I. Rodriguez* , Y. Metz and R. Voges

Preclinical Safety-Drug Metabolism, Isotope Section

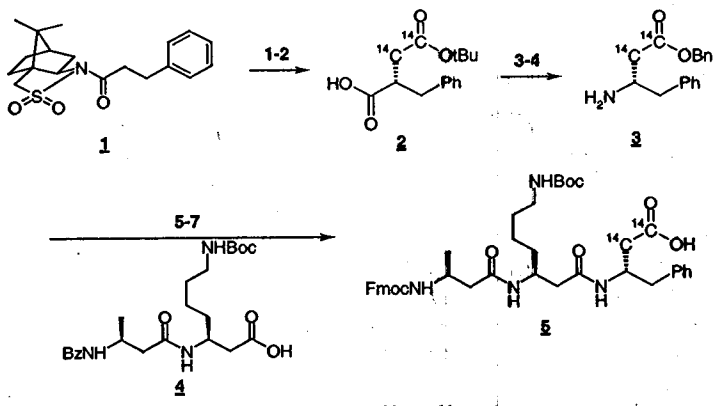
Novartis Pharma AG, CH 4002 Basel, Switzerland

* Internet: ines.rodriguez@pharma.novartis.com

There is considerable interest in enantioselective synthesis of β-amino acids since they are components of a wide variety of naturally occurring biologically active molecules as the well-known β-lactam antibiotics and several peptidic natural compounds with pharmacologically interesting properties [1]. More recently β-amino acids have proved to be useful building blocks in the synthesis of modified peptides which are potentially biologically active compounds exhibiting remarkable stability toward proteolytic processes [2].

It was planned to investigate the biological activity *in vivo* as well as the pharmacokinetic behaviour of a series of nona-β-peptides with potential antibacterial activities [3-4]. Having as a target β-nonapeptide **9**, the carboxy-protected β-amino acid fragment **3** ((S)-3-amino-4-phenyl-[1,2-$^{14}C_2$]butyric acid benzyl ester) was prepared via diastereoselective α-alkylation of the chiral acyl imide **1** with commercially available *tert*-butyl bromo[1,2-$^{14}C_2$]acetate, LiOH-mediated hydrolytic cleavage of the auxiliary, Curtius degradation of the free carboxyl group to the corresponding amine, and conversion of the tert-butyl ester into the respective benzyl ester (scheme 1)[5].

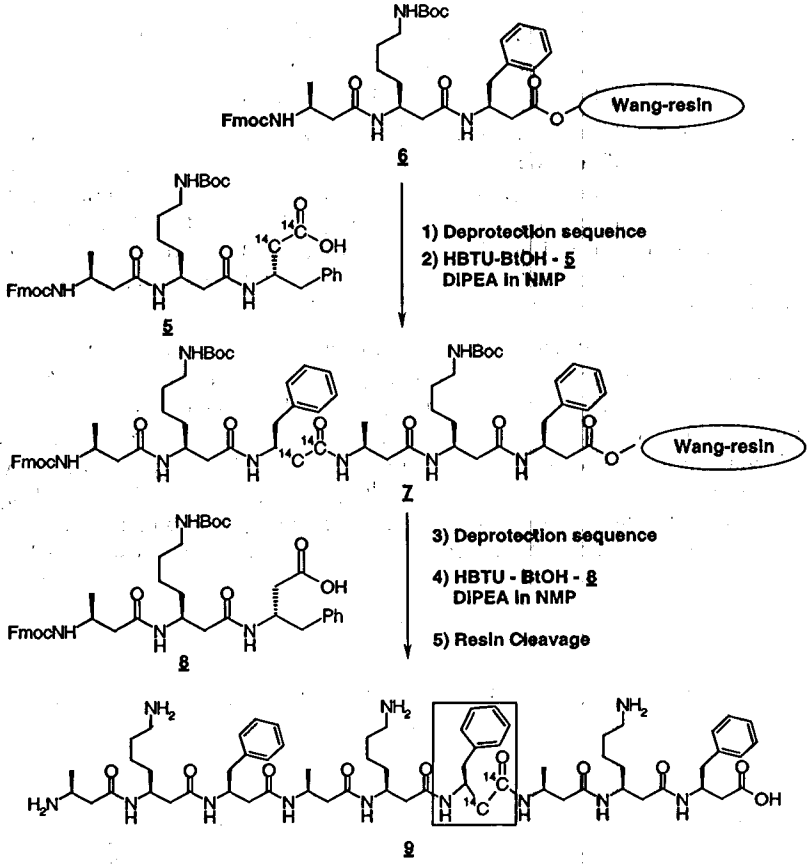
Scheme 1



Reaction conditions: 1) NaHMDS, THF-HMPA, $Br^{14}CH_2{}^{14}CO_2{}^tBu$, -78°C, 2h, 62%; 2) LiOOH, THF-H_2O, 0°C, 4h, 98%; 3a) $PO(OPh)_2N_3$, Et_3N, toluene, reflux, 2h; 3b) HCl 6N, dioxane, reflux, 1h; 4) Me_3SiCl, BnOH, rt, 16h 85%; 5) BtOH, EDC, CH_2Cl_2, rt, 16h, 46%; 6) H_2, Pd/C, CF_3CH_2OH, rt, 20h, 100%; 7) FmocOSu, dioxane, rt, 20h, 69%

Amino acid **3** was coupled to dipeptide **4** and the resulting tripeptide **5** subsequently incorporated into nonapeptide **9** via solid phase synthesis [6] (scheme 2) following routine procedures.

Scheme 2



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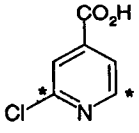
A Rapid Synthesis of 2-Chloro[2,6-^{14}C]isonicotinic Acid

Terence P. Kelly, Thomas R. Rodgers and Christopher Wright*

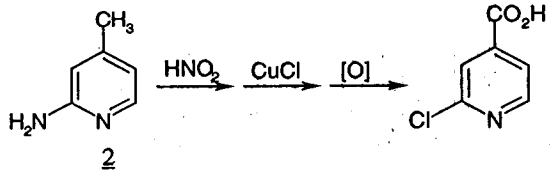
PerkinElmer Life Sciences, Inc.
549 Albany Street
Boston, MA 02118-2512
U. S. A

Field studies required that the title compound (1), a precursor for a ^{14}C-labeled herbicide, be synthesized and delivered rapidly. No short synthesis of this [^{14}C]compound has previously been reported.

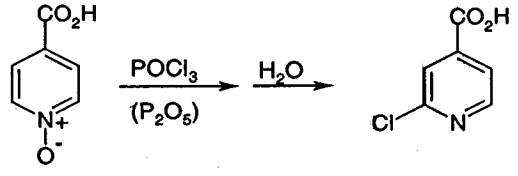


1

Previous syntheses of 2-chloroisonicotinic acid have been based on pyridine precursors, not readily available with a ^{14}C label (e.g. 2), or else on isonicotinic acid N-oxide (3); a compound which is prepared with a ^{14}C ring label by a somewhat lengthy and tedious synthetic method.

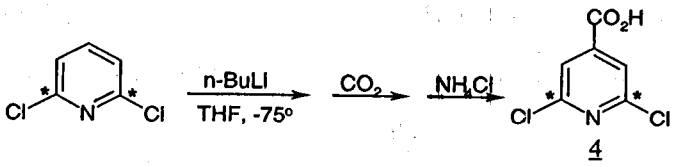


2

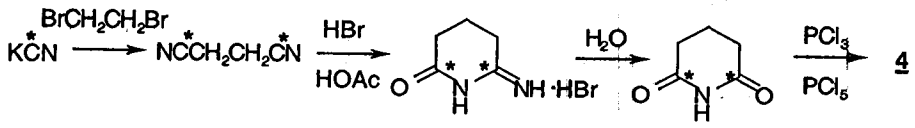


3

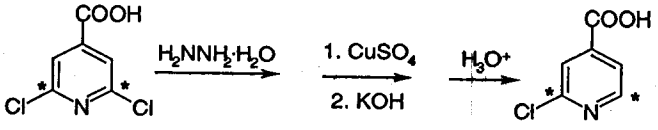
The title compound was obtained conveniently by lithiation of readily available 2,6-dichloro[2,6-^{14}C]pyridine 4 under careful kinetic control [1], followed by carboxylation:



The requisite 2,6-dichloro[2,6-^{14}C]pyridine 4 was conveniently available from potassium [^{14}C]cyanide in 4 steps in 73% overall radiochemical yield [2,3].



Subsequent selective mono-dechlorination of 4 with hydrazine hydrate by a literature procedure [4] followed by heating with aqueous cupric sulfate and potassium hydroxide produced the desired product, 99% radiochemically pure after chromatographic purification, in 22% overall radiochemical yield.



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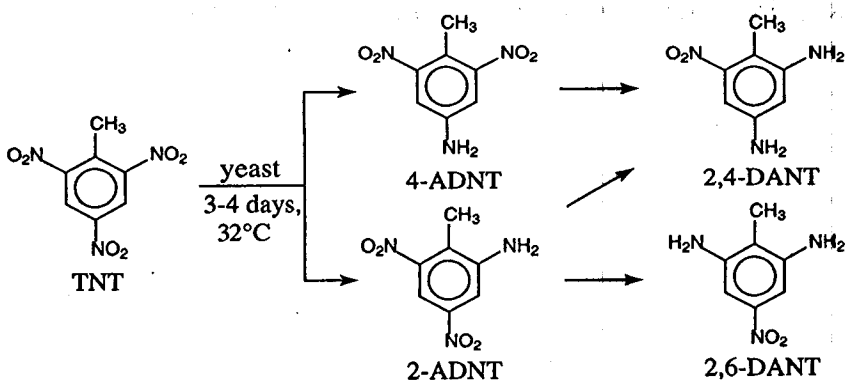
Reduction of 2,4,6-trinitro[ring-^{14}C]toluene (TNT) with baker's yeast (*Saccharomyces Cervisiae*)

Mario Kröger* and Gregor Fels

Universität Paderborn, FB 13 - Organische Chemie,
Warburger Str. 100, D-33098 Paderborn,
*MPK@Chemie.uni-paderborn.de

TNT is an important explosive and was produced in huge amounts during the last century, especially during the world wars. The toxic, cancerogenic and mutagenic properties of **TNT** as well as the chemical persistance have turned it into a major environmental hazard and the biological degradation is an ongoing challenge [1]. The reduced derivatives of **TNT**, aminodinitrotoluenes (**ADNTs**) and diamino-nitrotoluenes (**DANTs**), are ocurring as natural metabolites and their reactivity results in a higher toxicity compared to **TNT** [2]. On the other hand, these compounds are more susceptible to degradation reactions, and there is a growing interest in their role as possible intermediates in the conversion of **TNT** into commercially interesting derivatives [3,4].



For the reduction of **TNT** baker's yeast (*Saccharomyces cervisiae*) was found to be the microorganism of choice because of cheapness, availability and easy handling.

The enzymes of this whole cell system are able to reduce **TNT** to **DANTs** via OH-**ADNT** and **ADNT** intermediates. Despite of some variations in the regioselectivity,

formation of **4-ADNT** was generally favoured over **2-ADNT** and far more **2,4-DANT** than **2,6-DANT** was produced.

Optimization of the enzymatic reaction resulted in a one-step synthesis of **4-ADNT**, **2-ADNT**, **2,4-DANT** and **2,6-DANT** with overall yields up to 65%. Separation of the products is easily achieved by preparative TLC-techniques. Employment of [ring-^{14}C]TNT [5] in this procedure yielded the corresponding radioactively labelled materials.

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Application of Microbiotic Methods for the Synthesis of Radiolabelled Isotopomers - A Review [1]

Marion Mahnke [1], Thomas Moenius [2]*
[1] NOVARTIS Pharma AG, Pharma Research, CH-Basel
[2] NOVARTIS Pharma AG, Preclinical Safety, DMPK-IL, CH-Basel

Despite numerous synthetic methods available [2] which enable the introduction of a radiolabel, in the case of highly complex molecules of natural origin there still remains substantial limitations. Enzymatic (= cell free) and microbiotic (= cell containing) methods, however, are useful tools in which to overcome some of these problems. To date there are two comprehensive papers which document this. Simon [3a] provides a general overview of this subject, whereas Figdor [3b] focusses on methodological problems of the fermentation of the antibiotic compound salinomycin.

This presentation describes some later applications of microbiotic methods for H-3 and C-14 labelling that have since arisen in the literature. Addition of suitable radiolabelled building blocks to fermentation broths produces labelled metabolites and therefore represents an interesting biological alternative for the synthesis of radiolabelled isotopomeres. This review illustrates not only the synthetic potential of fermentation methods, but also their limitations.

Desisive for the outcome of the „radiofermentation" is not only type and productivity of the microorganism used, but also the kind of labelled building block, the composition of the fermentation broth and the set-up of fermentation parameters. Therefore the optimal study design requires quite a number of pre-experiments. When starting from a given type of microorganism biogenesis of the metabolite, temperature- and concentration dependency of the process, the inhibition of the microorganisms to the substrate and the sensitivity of such microorganisms to radiation have to be studied.

Based on this strategy compounds like geldanamycin [4], cyclosporn A [5], the everninomicin derivative SCH27899 [6], avermectin [7] and rapamycin [8] have sucessfully been labelled.

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SCREENING OF STORAGE-STABILITY OF ^{14}C RADIOCHEMICALS

Andreas Fredenhagen

Syngenta Crop Protection AG, Isotope Laboratory, 4002 Basel, Switzerland

andreas.fredenhagen@syngenta.com

Abstract: Storage of radiochemicals is a significant practical problem. Storage as a solution in various solvents was compared to the storage as neat (oil or solid) over an extended period of time. Addition of methylsulfide or 2-methyl-2-buten was shown to improve the stability by a factor of 1.7 to 3.2 in ethanol-free solvents mixtures, Thioethers are inert and enhance the storage stability considerably. General points to consider for storage of radiochemicals will be discussed.

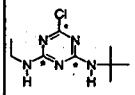
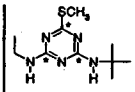
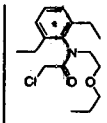
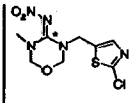
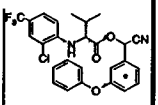
Due to internal irradiation decomposition of labelled compounds occurs much faster than for the non-labelled compound and so frequent re-purification and re-analysis are required. These time-consuming procedures can be reduced by suitable storage. It was observed some time ago that storage in solution may reduce the rate of decomposition of labelled organic compounds [1].

Compounds, whose degradation rate as solids exceeded 0.5 % per month, were selected for the present study. A simple screening procedure was set up to test for stability of newly available compounds: 0.7 mg of material was transferred into a 1.5 ml flask and dissolved in 200 μl of the appropriate solvent mixture. Radiochemical purity was determined by HPLC equipped with a solid-phase radiodetector.

The results of storage of various solutions were compared with the storage as neat compounds (Table 1) . Oily compounds degrade to a purity below 95% within a few weeks if not stored as solutions. For all compounds investigated the storage in solution proved to be best. However the best choice of solvent depended on the compound and is not predictable. For compounds which are soluble in most solvents and where no reactions with solvents are expected toluene / ethanol - mixtures are usually favorable. Tau-fluvalinate, however, degrades in the presence of alcohols.

From the observation that **2** is considerably more stable in solutions of dichloromethane than the closely related compound **1**, it was postulated that sulfur compounds might be beneficial in interrupting radical chain reactions involved in the degradation of radiochemicals. Therefore methylsulfide, 2-mercaptoethanol, 2-methyl-2-buten and benzyl alcohol were investigated as stability enhancers. In toluene/ethanol mixtures no effect was observed. For all other solvent mixtures investigated addition of methylsulfide increased the stability, most notably in the solvent mixtures containing dichloromethane. Addition of 2-methyl-2-buten was beneficial for the storage of the triazines **1** and **2**. Benzyl alcohol was in no case superior to other stability enhancers. It is noteworthy that addition of methylsulfide is always beneficial, and no case of decreased stability has been observed.

Table 1: Storage stability of radiolabelled compounds as neat and in solution

Storage conditions [a]	% Decay Per Month				
	 **1** terbuthylazine	 **2** terbutryn	 **3** pretilachlor	 **4** thiamethoxam	 **5** tau-fluvalinate
Physical property	solid	solid	oil	solid	oil
Specific activity (MBq /	7.5	6.9	5.3	3.0	2.0
Concentration (mg/ml)	2.5	3.5	5	5	6
Period observed	12.7	12.6	19	6.4	25
Neat, -20 °C	0.97	1.05	7.9	0.82	1.4
In acetone	0.32	0.66	0.36 [d]		
In acetonitrile			0.61	0.56	0.41
In benzene or toluene	0.58 [g]	0.46	0.69 [e]		0.48 [e]
In benzene / CH_2Cl_2 / methanol 1:1:1	0.94 [g]	**0.27**			
In dichloromethane				0.94	
In CH_2Cl_2 / MeOH 1:1	1.2	0.21		**0.063**	
In methanol [a]			0.68	0.23	
In toluene / ethanol 95:5	0.21	0.24	**0.38** [f]		
Solvent used	C_6H_6 / CH_2Cl_2 / MeOH 1:1:1 [h]	C_6H_6 / CH_2Cl_2 / MeOH 1:1:1 [h]	toluene / ethanol 9:1	CH_2Cl_2 / MeOH 1:1 [h]	toluene
Specific activity (MBq /	2.0	2.6	5.3	2.2	2.0
Concentration (mg/ml)	10	16	5	15	6
Period observed	9	27	19	27	25
Without addition	0.41 [g]	0.21	0.38	0.13	0.48
+ 2-methyl-2-buten [b]	**0.13** [g]	0.11	0.37	0.11 [c]	0.39
+ methylsulfide [b]	0.18 [g]	**0.08**	0.42	**0.07** [c]	0.42
+ 2-mercapto-ethanol	0.24 [g]	**0.08**	0.46	0.11 [c]	0.28 [i]
+ benzyl alcohol [b]	0.21 [g]	0.16		0.10 [c]	
Improvement factor	**3.2**	**2.6**	**0**	**1.9**	**1.7**

a) all at - 20 °C, b) 2% (v/v) addition c) 1% (v/v) addition
d) acetone / benzene 95:5 e) toluene f) toluene / methanol 90:10
g) degradation is accelerated 2 to 3-fold after 27 months (non-linear decomposition)
h) dichloromethane - mixtures chosen for solubility reasons
i) not suitable: material decomposes upon evaporation of solvent
* denotes the position of the [^{14}C]-label

Acknowledgments

Thanks are due to P. Thür for skilled purification and to Dr. P. Ackermann for helpful discussions.

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APPLICATION OF AMS FOR HUMAN ADME

[1] F. Waldmeier, [2] R. C. Garner

[1] Novartis Pharma AG, DMPK, WKL-135.226, CH-4002 Basel, Switzerland
[2] CBAMS Ltd, Sand Hutton, York YO41 1LZ, UK
Felix.Waldmeier@Pharma.Novartis.com

SUMMARY

The use of AMS for human ADME will be discussed and exemplified with an „Early Human ADME Study".

KEY WORDS

AMS, Accelerator Mass Spectrometry, ^{14}C, ADME, Pharmacokinetics, Human, Phase I, Drug Development, Regulations, Radiation Exposure, Dosimetry

AMS

AMS (Accelerator Mass Spectrometry) is the most sensitive detection system for the ^{14}C-isotope. In biological samples, ^{14}C-amounts of <0.01 dpm may be detected. AMS is commercially available for analysis of human samples from drug studies.

HUMAN ADME

A key study in drug development is the human ADME study (absorption, distribution, metabolism, excretion). Typically, it is performed in clinical phase I or II using tolerable mg-doses, and ^{14}C-radioactivity doses of 30-100 μCi. International guidelines and national regulations require prognostic dosimetry calculations based on animal ADME and human PK data. From the dosimetry, the radioactivity dose is derived at which radiation exposure is below legal limits (ICRP and CH: 1 mSv). For analysis of biological samples, commercial equipment like LSC, HPLC-radiomonitors (on-line), LC-MS(-MS) and NMR are used routinely.

NEW OPPORTUNITIES WITH AMS

A classical ADME study may not be feasible if radiation exposure is too high due to high organ concentrations or long retention, or with special dosing routes. In early development it is not feasible since several prerequisites are likely not to be available. However, when biopharmaceutical properties of a drug are pivotal, an early human ADME study would be useful. With AMS, early human ADME studies are feasible, as ^{14}C-levels in human samples can be detected after extremely low ^{14}C-doses.

EARLY HUMAN ADME STUDY

We have conducted an early human ADME (1st-into-man) study with a drug candidate for which absorption, bioavailability and metabolism rate were key issues. The trial was designed as a pilot, dose-escalation / ADME / PK / tolerability study. Single, oral, subpharmacological doses of 1, 5 and 10 mg drug, containing 50, 250 or 500 nCi ^{14}C were administered to healthy volunteers (N=2 each). Doses were safe based on Tox studies in three animal species. Dosimetry calculations, according to ICRP and CH regulations, based on animal ADME/ PK data and allometric scaling to man, showed that the radiation exposure („effective dose") would be <10 μSv (CH limit). Drug substance of high specific radioactivity was analysed and released for human use. Final drug substance of low specific radioactivity was prepared by dilution with unlabelled GMP drug substance and adequately analysed for release. The drug was administered in a freshly prepared aqueous drink solution. The protocol was approved by the Ethics Committee, the Swiss HA, and the Swiss Radiation Protection Authority. The latter requested an application including dosimetry.

Blood, urine and faeces samples were collected. Plasma was analysed for parent drug, and ^{14}C-levels were measured by AMS. In excreta, radioactivity was measured using LSC. The metabolite pattern in faeces extracts was analysed semiquantitatively using HPLC with off-line LSC counting. A metabolite pattern in plasma, after protein precipitation, was obtained by HPLC with AMS analysis of fractions.

RESULTS AND DISCUSSION

In plasma, dose-related ^{14}C-levels were detected, demonstrating approximately dose-linear absorption. Parent drug levels were below LOQ. A large dose proportion was excreted within 3 days with faeces. Fecal metabolite patterns showed little unchanged drug but a large number of metabolites. A similar pattern was found in plasma. The results demonstrated that the drug candidate was well absorbed, but was subject to extensive metabolism, probably at the first liver passage (1st pass).

CONCLUSION

With the presented early ADME study key information on the drug candidate was obtained rapidly. The study was simple and partly semiquantitative, but adequate for fast decision making. The example shows how an early human ADME study can be performed, and illustrates the usefulness of AMS in early drug development.

ACKNOWLEDGEMENT

The authors want to express thanks to a number of colleagues from several departments of Novartis, particularly to Dr. H.Andres (Isotope Lab).

BINDING STUDIES FOR AFFINITY SCREENING TO THE EXSORPTIVE TRANSPORTER P-GLYCOPROTEIN: [^{3}H]TALINOLOL AS RADIOLIGAND

Peter Langguth[1], Annette Koggel[1], Cornelia Dressler[2], Günther Peinhardt[2], and Hildegard Spahn-Langguth[2]

[1] School of Pharmacy, Johannes Gutenberg-University, Staudingerweg 5, D-55099 Mainz
[2]Department of Pharmaceutical Chemistry, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle/Saale
langguth@mail.uni-mainz.de

The intestinal absorption, but also the disposition of xenobiotics may be affected by the occurrence of absorptive or exsorptive transporters in the cellular membrane. In this respect the exsorptive ATP-driven transport mediated by P-glycoprotein (P-GP; ABC transporter) is currently extensively investigated. In addition to the frequently applied uptake or efflux assays or transport studies through monolayers, a radioligand binding assay was developed that permits P-GP affinity screening of non-labelled compounds via competitive inhibition of radioligand binding.

The assay is based either on MDR1-transfected or vinblastine-induced P-GP-overexpressing Caco-2 cells and cell poration with lysolecithine [1]. Various P-GP substrates were tested for their suitability as radioligands - including studies on nonspecific binding (e.g., to filters or cell components) and the specific-to-nonspecific binding ratio -, among them [^{3}H]vinblastine, [^{3}H]verapamil, and [^{3}H]-talinolol (bi-tritiated) (Figure 1) [2].

Particularly because of its low nonspecific binding [^{3}H]talinolol showed the most favourable properties among the tested potential radioligands and was, hence, chosen for future studies. Binding studies yielded typical inhibition curves, for which inhibitory constants were calculated. The assay showed good variability characteristics and reproducibility, unless the respective test compound was poorly soluble in aqueous solutions. Typical IC_{50} values (at 1 μM radioligand) as obtained with this assay were talinolol, 523 μM; alprenolol, 436 μM; and verapamil. 341 μM (Figure 2).

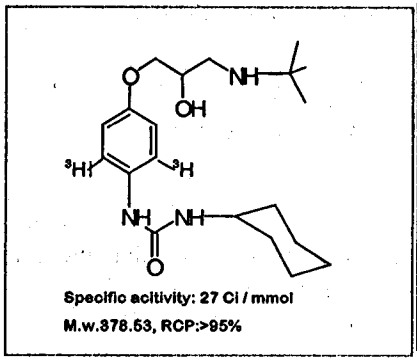

Figure 1: [^{3}H]Talinolol

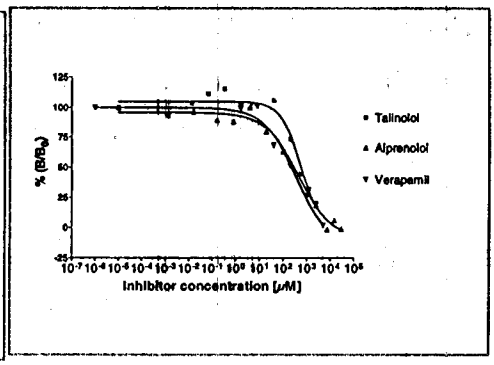

Figure 2:
Inhibition curves obtained from the P-GP RBA (corrected for non-specific binding)

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STABLE ISOTOPES IN MEDICINE

G.E. von Unruh
Medizinische Klinik und Poliklinik I, Universitätsklinikum Bonn, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany

Summary
Compounds labelled with stable isotopes were used as diagnostic agents. The most frequently applied test at present is the [^{13}C]urea breath test for presence or absence of *helicobacter pylori* in the human stomach. Function tests for digestion, absorption and excretion were developed and produced relevant research information, but were not yet applied on a routine basis. Bioavailability studies and pharmacokinetics of interacting drugs or drug enantiomers in patients under long term therapy were other small, but important fields of medical application for stable isotope labelled drugs.

Key words: stable isotope, drug, pharmacokinetics, diagnostic agent, oxalate

Introduction
Drug molecules applied at different times or by different routes were indistinguishable, differentiation of drug enantiomers and their metabolites was difficult without isotopic labelling. The source of compounds with dual origin (e.g. endogenous metabolite versus drug metabolite or endogenous metabolite versus natural component of food) was indistinguishable without isotopic labelling. In other words, an isotopic label can serve as a label for time, for a galenic formulation, for a means of application, for the stereochemistry or for the precursor even after a series of complex biochemical reactions.

Results and Discussion
The [^{13}C]urea breath test for presence or absence of *helicobacter pylori* in the human stomach emerged as the most prominent member of a series of breath tests (1). The health insurance paid for this test, leading to a widespread acceptance by the medical community. A specific reaction – the cleavage of labelled urea by the urease of *helicobacter* in the stomach in this case – liberated the labelled carbon atom as CO_2. Because of the label, this CO_2 could be detected within the CO_2 exhaled coming from dozens of other sources simultaneously (2). The arguments in favor of breath tests:
(i) ease of obtaining the material to be analyzed (no blood sampling, no endoscopy),
(ii) ease of automatic work-up of breath samples as compared to a matrix like blood,
(iii) relatively cheap dedicated analytical equipment.

Examples for clear-cut advantages of the use of stable isotope labelled drugs were pharmacokinetic studies in patients requiring uninterrupted therapy, like in the case of patients suffering from heart problems or epileptic seizures. In the last group of patients, like in very old people, polypharmacy was normal. Multiple drugs, especially in combination with impaired body functions (heart, liver, kidney) lead to greatly altered metabolism and excretion of drugs. Drug interactions, seldom to an advantage for the patient, were the rule. Most antiepileptic drugs, especially carbamazepine,

phenobarbital and phenytoin induced the synthesis of liver enzymes responsible for oxidative degradation, thereby acceleration their own elimination (up to fivefold) and that of other endogenous and exogenous compounds (3, 4). We also studied the pharmacokinetics and metabolism of the enantiomers of tramadol (5), defining pharmacogenetic differences and identifying a new hydroxymetabolite.

The [$^{13}C_2$]oxalate absorption test is a newer example of a function test (6, 7). In animals including man oxalate is a metabolic end product – no further metabolism takes place. In a large number of our normal food plants it is also a terminal metabolite and serves further for the plant as a protective poison against herbivores. In humans calcium oxalate is the main component of most urinary tract stones. Knowledge of the origin of oxalate is therefore required for a rational therapy against calcium oxalate urinary stones.

Experimental
The analysis was by mass spectrometry, for details see the literature cited.

Conclusions
Compounds labelled with isotopes made the research easier in a large variety of medical problems. Furthermore, labelled compounds allowed several measurements that were impossible without isotopic labelling as the measurement of single dose kinetics during uninterrupted therapy or source or precursor measurements with common terminal end products. Stable isotopes were preferred because patients, volunteers and staff feel easier, there was less bureaucracy and for certain groups radioactive isotopes were not acceptable by the ethics committees. Radioactive isotopes were preferred because of the ease of detection. These days, radioactive isotopes were seldom used in medicine outside of the departments of nuclear medicine (ignoring the use of natural abundance ^1H for magnetic resonance tomography).

Acknowledgement
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GMP-TRENDS FOR CLINICAL TRIAL SAMPLES

R. Schnettler*, C.R.Oechslein

PTS Training Service, P.O. Box 4308, D-59737 Arnsberg, Germany
mailto:info@pts-aktuelll.de

Summary:
During Development of new drugs it may be necessary to perform phamacokinetic/Ppharmacodynamic studies with radiolabelled medications in humans. In contrast to similar studies in lab animals, for the preparation, control, and documentation of clinical trial samples additional quality standards apply: all pharmaceutical preparations intended for human application must be prepared according to GMP.

Key Words: Good Manufacturing Practices, Risk Assessment, Qualification, Traceability, Documentation, Cross Contamination Prevention,

Introduction:
It is a matter of fact, that control and release of raw materials together with control and release of finished pharmaceutical products may not necessarily result in products meeting the required quality and safety level. Certain aspects of the manufacture are crucial for the product quality, efficacy, or safety but may not be detectable by analytical means.
Instead, in order to achieve a product which is highly likely to meet the specifications at the end control, the manufacturing process itself needs to be well controlled, the manufacturing area must be monitored, the equipment is to be qualified, calibrated and maintained, the personnel should be qualified and continuously trained and documentation must allow traceability of ingredients and intermediates. All these aspects are covered and regulated by GMPs. These guidelines, firstly published by WHO in 1968 for pharmaceutical products, became regulatory requirements in all major countries over the recent years. They are currently applied also to active pharmaceutical ingredients, excipients and clinical trial medications.

Discussion:
For preparation of radiolabelled clinical supplies the following basic GMP requirements are extremely important:

- Written manufacturing and control instructions, authorized by a Quality Unit must be strictly followed; all deviations must be cross-checked for influence on GMP-status
- personnel must be trained to perform and document the process exactly
- specifications must be defined for IPC and end product controls
- procedures must be defined, what to do, when specifications are not met
- it must be shown, that cleaning procedures applied reproducibly lead to a tolerable residual contamination

- all facilities and equipment involved must be part of written cleaning, monitoring, maintenance, qualification and calibration plans
- documentation must allow complete traceability of products and ingredients upstream and downstream. Identification of raw materials used must be traceable through e.g. a batch numbering system. The finished product must be traceable to the patient and retrievable in case of severe side effects.
- if packaging and shipment of labelled test samples are done under quarantine, there must be a procedure assuring that medications may not be used prior to quality control and release

In early phases of clinical development, when manufacturing of clinical trial medications is limited and continuously changes practicability of the implementation of a GMP system may be questionnable. Nevertheless, apart from regulatory requirements there are more good reasons to comply with GMP already in this stage:

1) the safety and well-being of patients must be assured
2) quality and reproducibilty of the whole clinical study depends on the quality and reproducibility of the clinical trial samples administered
3) the quality of the formulations tested in clinical studies must be equivalent to the quality of the (planned) commercial product

Finally, GMP requirements increase as the development process progresses. In early stages authorities accept a limited Quality Assurance concept, provided, the risk is assessed individually: e.g. in cases, where GMP principles cannot be applied, e.g. for synthesis of isotopically labelled compounds, additional control steps must be performed.

Conclusion:

There are lots of regulatory requirements and guidelines dealing with GMP aspects in all stages of manufacturing and control of Drug Substances, Excipients, Drug Products and Clinical Supplies, but there is no universally adoptable concept which fits all. Therefore it is up to the expertise of the pharmaceutical manufacturer *how* to comply with specific requirements. For manufacturers of drug substances, especially of radiolabelled compounds, the authorities leave the door open to individual solutions, provided that scientific sound principles are used and documented, such as hazard analysis.

References:

EG Guide to GMP, Annex 3
FDA Guideline on the preparation of investigational new drug products, 3/91
PIC Supplementary Guideline on GMP for investigational pharmaceutical products PH6/93
EEC Guideline "GMP for Investigational Medicinal Products" III/3004/91-EN
ICHQ7A, § 19 "APIs for Use in Clinical Trials"

CHANGES IN RADIATION PROTECTION IN LAW IN GERMANY

H.-J. Reinecke
University of Tübingen, Germany
hans-juergen.reinecke@uni-tuebingen.de

Summary

Regarding to the Council Directive 96/29/Euratom of 13 May 1996 laying down basic safety standards for the protection of the health of workers and the general public against the dangers arising from ionizing radiation the German Atomic Law (Atomgesetz) and Radiation Protection Decree (Strahlenschutzverordnung) should be repealed with effect within 4 years from the date that this directive became applicabled. The Atomic Law is already amended and there is now a draft of the Radiation Protection Decree avaible having regard to the German Federal Government but not yet to the county governments.
In the new draft natural radiation sources from natural terrestrial or cosmic origin are included which lead to a new structure of the "Strahlenschutzverordnung". Part 1, 2, and 5 and parts of the appendices mainly include the last Radiation Protection Decree. Part 3 and 4 are new parts.

Part 1	General directives Aim in view, area of application, definitions
Part 2	Practice Protection for population and environment at the practice of radioactive substances or ionizing radiation
Part 3	Work *Protection for population and environment from natural radiation sources*
Part 4	protection for the consumer *protection for the consumer, when radioactive substances are added to products*
Part 5	Common directives consideration of radiation exposures, authorization of the competent authority, regulations for documentation, „Ordnungswidrigkeiten", regulations at the end
Appendices	I - IVX

In this lesson only part 1, 2 , 5 and the Appendices are important and the most important details are reported.

In the draft there are lower dose limits than in the last "Strahlenschutzverordnung" and they are
usually more strictly than the Council Directive 96/29/Euratom, having regard to the opinion of
the county governments, not to decrease the standards of protection of the last one. In the next chart some new dose limits are shown (former values in brackets).

object	dose limit (former dose limit)
Effective dose for exposed workers inclusive Categorie A	< 20 mSv/a (< 50 mSv/a)
Effective dose for exposed workers Caterogy B	< 6 mSv/a (< 15 mSv/a)
uterus for child-bearing women	≤ 2 mSv/m (< 5 mSv/m)
Protection of the child to be born	≤ 1 mSv during pregnancy ( )
Effective dose for persons younger than 18 years (apprentices and students)	< 1mSv/a (< 5mSv/a)
Effective dose for members of the public	< 1mSv/a (< 5 mSv/a)
Controlled area	6- 20 mSv/a (< 15-50 mSv/a)
Superwised area	1- 6 mSv/a (< 5-15 mSv/a)

In the future Medical surveillance usually is required only for exposed workers Caterogy A but not any more for Caterogy B. Women during pregnancy and breastfeeding are now allowed to enter Controlled areas, when they are not employed in work involving the risk of bodily radioactive contamination.

There are new exemption values of the quantity of activity and the concentration of activity per unit. Only the values of tritium 10 MBq (5 MBq) and carbon-14 1MBq (0,5MBq) are a factor 100 lower than the values of the Council Directive 96/29/Euratom but they are still in discussion.
In the new "Strahlenschutzverordnung" licensing ("Genehmigung") needs to be required if the quantities of radioactive substances involved in total and the concentration of activity per unit in total exceed the exemption values. Reporting ("Anzeige") is no longer included in the Radiation Protection Decree. In future it is not allowed to handle radioactive substances which in total do not exceed the exemption values without licensing, if these nuclides are already part of a licensing in the same place. This will lead to problems in the future.
The new exemption values now correspond to the new international exemption values for the transportation of dangerous goods, which are obligatory from 1. Juli 2001 onwards in Germany.

There are new values for contamination limits divided in five classes of nuclides contrary to former three classes. The limits mostly are upgraded by a factor of 2, 20 or 200.
To release from the requirements of the Atomic law new clearance levels for disposal, recycling, or reuse of radioactive substances or materials containing radioactive substances arising from any practice are established. These clearing levels are lower than the exemption values of the concentration of activity per unit or equal the values.
The information and training for exposed workers, apprentices and students in future will be necessary regularly once a year in contrast to half a year before. On the other side the qualified radiation expert ("Strahlenschutzbeauftragter") has to polish up his license ("Fachkunde")within 5 years.
In the draft there are some new definitions which are important for the understanding of the special paragraphs. The temporary regulations are established after the paragraphs of the "Strahlenschutzverordnung". They give a varied period of grace normally between 2 month and 5 years to apply induvidual rules.

INFORMATION SYSTEMS FOR RADIATION PROTECTION

J. Gay, Schering AG, Berlin, Germany
H. Graffunder, Ingenieurbüro Graffunder, Stutensee, Germany
juergen.gay@schering.de

Ingenieurbüro Graffunder offers MS-Windows based software packages for a width area of operative and administrative radiation protection.

The radiation protection information system *SISy* has approximaly 300.000 physical, chemical and radiological data for about 2.500 nuclides. Based on this data, various information concerning radiation protection can be extracted or calculated. *SISy* is a help- and information-software-system for responsible people in radiation protection.

RaQuel is a radioisotope stock control and tracking system. Beyond bookkeeping and the declaration of aquisitions to fulfill the licence requirements in terms of ordering, receiving and distribution, it is also a helpful tool for the handling of all kinds of radiocative compounds. Shipments and translocation of all radioactive samples or aliquot for internal or external sites are back-traceable. Each compound can be characterized by using more than 70 data fields in terms of authority demands, physical and chemial properties and their individual specification. *RaQuel* is also a quick and suitable tool for the inventory and the disposal of radiactive waste.

StraDos is a software tool for documentation and monitoring personal dose values and timelines for instructions and the medical supervision and other relevant data on supervised persons in accordance with the requirements from the authorities.

Other software products: *QuaSi* for monitoring radiation protection quality, *TraKSi* is a checking and documentation system for radioactive transport according to ADR Class 7, with *Nuklidvektor* it is possible to store nuclide vectors and calculate nuclide inventories, *StrlSchV, §20-Ratgeber* and *GGVS-Ratgeber* are electronic books with texts from law and practical advising.