Recl. Trav. Chim. Pays-Bas **112**, 191–199 (1993) 0165-0513/93/02191-09$06.00

Synthesis and characterization of isotopically labelled drugs

Frans M. Kaspersen, Jan F. Vader, Carel W. Funke and Eric M. G. Sperling

Organon International B.V., P.O. Box 20, 5340 BH Oss, The Netherlands
(Received July 20th, 1992)

Abstract. The article describes approaches for the synthesis and analysis of isotopically (^{2}H, ^{3}H, ^{13}C, ^{14}C, ^{35}S) labelled drugs necessary for the development of a pharmacologically active compound into a registered drug.
Special features such as isotope effects, radiolysis and ^{1}H NMR spectra are discussed.

Introduction

The development of a pharmacologically active compound into a marketable drug is a long process. Toxicological investigations and the assessment of clinical efficacy are the main topics but the determination of the fate of the compound in the biological system and its mechanism of action are also important issues. For the so-called A(bsorption), D(istribution), M(etabolism), and E(xcretion) studies, the drug labelled with an isotope is an essential tool. In this article we describe, using examples from current research programmes within our pharmaceutical company (Organon Int. BV), the synthesis and analytical characterization of such materials and discuss their application in the development programme of a pharmaceutical drug.

Application of labelled drugs

To be able to draw valid conclusions from studies with the labelled drugs, the labelled material should mimic the drug as closely as possible. Unless we are dealing with high molecular weight biologicals (such as monoclonal antibodies) this implies that only isotopic substitution can be applied, e.g. ^{2}H or ^{3}H for hydrogen, ^{13}C and ^{14}C for natural carbon and ^{35}S for natural sulphur[a]. For deuterated and tritiated drugs especially, the labelled positions should be selected in such a way that isotope effects are minimal. Two isotope effects are possible: the so-called kinetic isotope effect where as a result of labelling, the metabolic breakdown of the drug is changed or inhibited[1] [illustrated for the antidepressant drug mirtazapine (**1**) in Figure 1] and the thermodynamic isotope effect where, as a result of labelling, p*K*'s and/or hydrophobicity change[2,3]. Large isotope effects are observed for aliphatic amines labelled with deuterium or tritium at the α and β positions[4,5]. Using straight-phase HPLC, such labelled and unlabelled materials can be separated. As illustrated in Figure 2 we applied this isotope effect to prepare highly labelled [N-$C^{3}H_{3}$] mianserin **2** (an antidepressant drug) by removal of the non-labelled fraction.

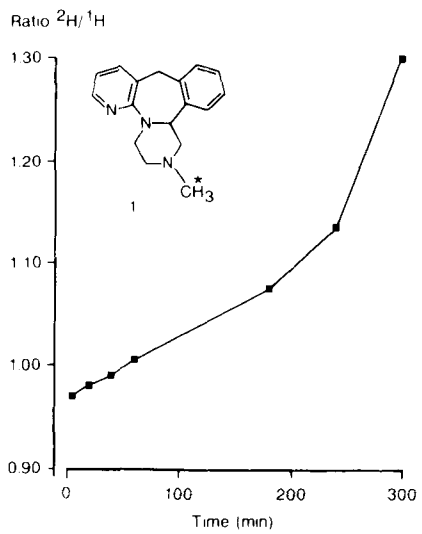


*Figure 1. Ratio of deuterated mirtazapine/unlabelled mirtazepine (**1**) in the plasma of Wistar rats after intravenous administration of 2.5 mg [N-$C^{2}H_{3}$]mirtazepine and 2.5 mg [N-$C^{1}H_{3}$]-mirtazepine[21]. This drug is cleared from circulation in rats by metabolic breakdown through N-demethylation.*

Deuterium-labelled drugs are mainly used as internal standards in GC/MS bio-assays; ^{13}C-labelled materials are used for the assessment of the bioavailability of drugs[6] and to study the enantiomeric aspects of pharmacokinetics of racemic drugs. As shown in Figure 3 for mirtazapine (**1**), a racemic drug where both enantiomers contribute to the pharmacological effect, a pseudoracemate consisting of 50% of one enantiomer labelled with ^{13}C and 50% of its unlabelled antipode was administered and the $^{13}C/^{12}C$ ratio

[a] ^{3}H, pure β emitter, maximum energy 0.0186 MeV; $t_{\frac{1}{2}}$ 12 years
^{14}C, pure β emitter, maximum energy 0.156 MeV; $t_{\frac{1}{2}}$ 5700 years
^{35}S, pure β emitter, maximum energy 0.167 MeV; $t_{\frac{1}{2}}$ 88 days

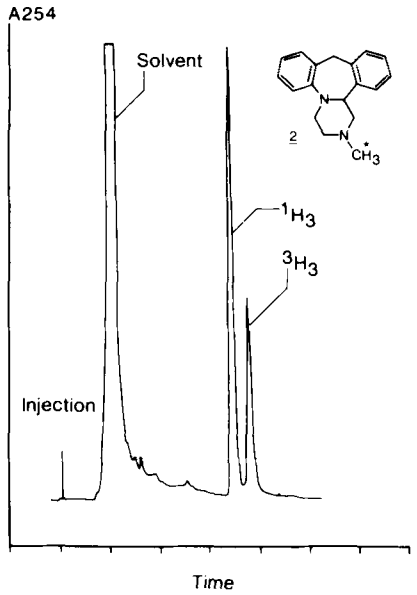


Figure 2. HPLC chromatogram of [N-C$^3H_3$]mianserin (**2**) *contaminated with [N-C$^1H_3$]mianserin. Starting material ≈ 1.0 tritium atom/molecule; purified material: 2.9 tritium atoms/molecule. HPLC conditions: Lichrosorb Si60 with n-hexane/propanol-2/aq · NH_4OH as eluent.*

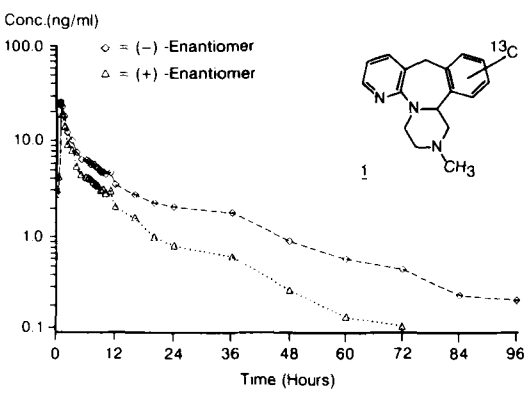


Figure 3. $^{13}C/^{12}C$ ratio in the plasma of a male volunteer after oral administration of 30 mg of a pseudoracemate of mirtazepine (**1**) *consisting of 50% of one enantiomer labelled with ^{13}C and 50% of its unlabelled antipode*[22].

of the drug was measured as a function of time with a GC/MS assay.

Drugs labelled with tritium and carbon-14 are used for the assessment of metabolic pathways and excretion of drugs in laboratory animals and man. The isotope and the site of labelling should be selected in such a way that maximum information about metabolic degradation can be obtained but for some drugs, *e.g.* the anti-angina drug bepridil (**3**), we had to apply three different labelled compounds to unravel the complex and extensive metabolic pathways[7] (Figure 4). For mechanism of action studies (*e.g.* receptor-binding studies), tritiated materials or drugs labelled with ^{35}S are often used. For studies with radioactive materials, only small quantities are necessary: less than 1 µg for ^{35}S, less than 100 µg for ^3H and up to 10 mg of the ^{14}C-labelled compounds: on the other hand for ^{13}C- and ^2H-labelled materials, up to gram amounts are usually synthesized.

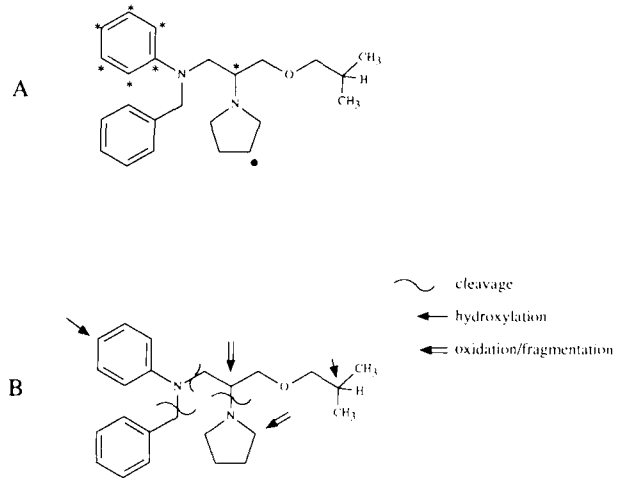


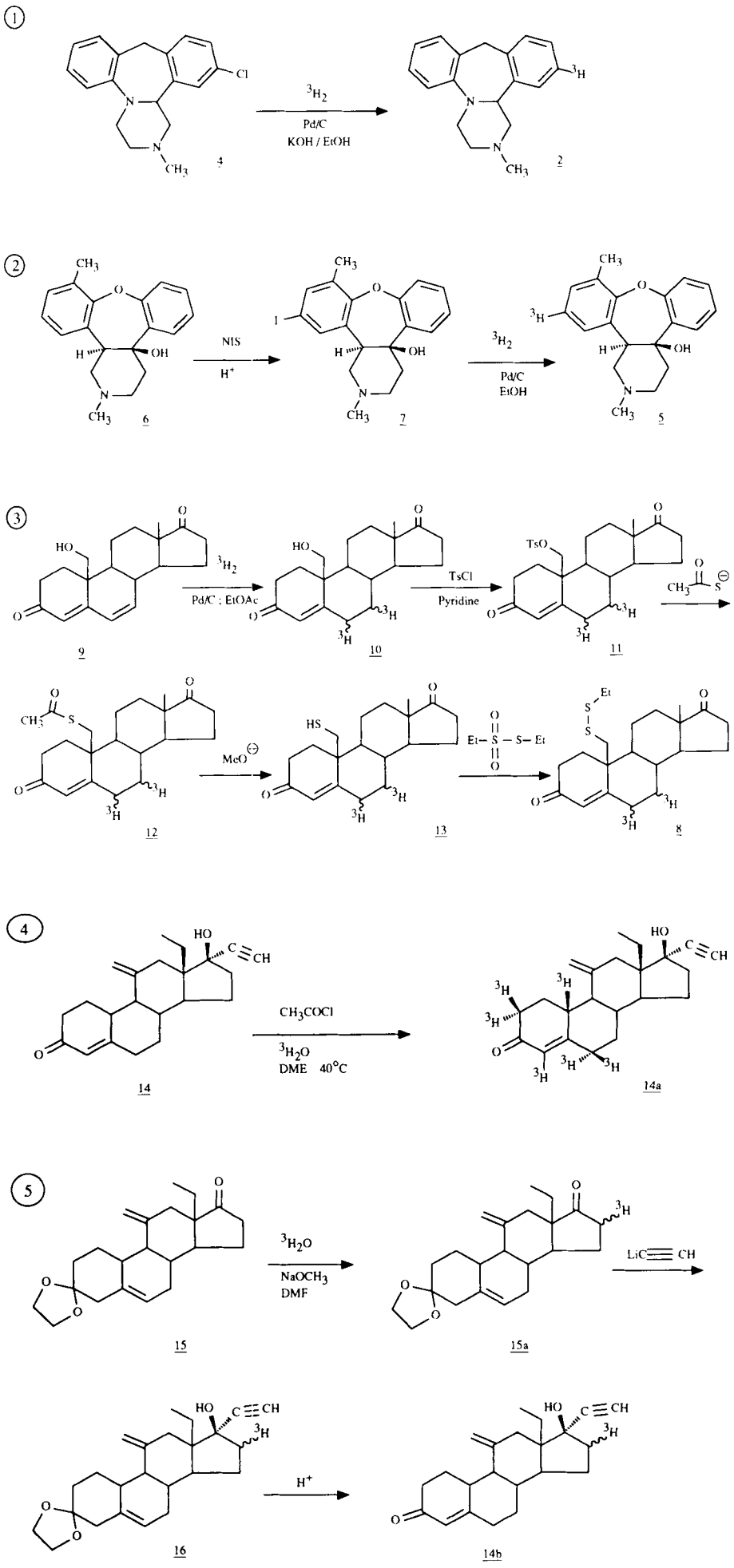
Figure 4. A: radioactive bepridils ([pyrrolidine-^3H]-, [β-propyl-^{14}C]- and [N-phenyl-U-^{14}C]-bepridil) used in metabolic studies; ● = ^3H · = ^{14}C. B: metabolic pathways of bepridil.*

Synthesis of isotopically labelled drugs[a]

The most common and inexpensive reagents used in the synthesis of tritiated molecules are tritium gas and tritiated water. With tritium gas, the ^3H is introduced, either by reduction of double bonds, triple bonds, reductive dehalogenations or by benzylic/allylic exchange, whereas for tritiated water, acid- or base-catalysed exchange reactions are applied[8]. Reduction with tritides is also possible; NaB^3H_4 is commercially available, whereas more reactive reductive agents like $LiEt_3B^3H$, selectride and also the tin tritides can be prepared *in situ* with Li^3H[9]. Some examples are given in Figure 5.

For heterocyclic compounds active in the central nervous system, such as mianserin (**2**) (entry 1) and the potential antidepressant Org 4428 (**5**) (entry 2), tritium is usually incorporated through reductive dehalogenation in the presence of Pd/C as catalyst. As starting material we use either chloroaromatic derivatives that are quite often available from series prepared for structure/activity relationship studies or iodo derivatives that are prepared through electrophilic iodination using *N*-iodosuccinimide and trifluoro methanesulphonic acid[10]; because of the strong acidic reaction conditions no interference is observed with the amine part of the molecules while the iodination species formed are very reactive. While tritiation of iodo derivatives proceeds smoothly (reaction time 30 minutes) the chloro derivatives have to be activated by the addition of a base; when a 1% solution of KOH in ethanol is used, reaction times of 1–2 hours are sufficient for complete conversion. In the absence of base, selective deiodination or debromination can be performed in the presence of chlorine substituents. Tritiated estrogens can be prepared by benzylic exchange while (nor)testosterone analogues and their derivatives are often synthesized by reduction of the 6–7 double bond. In the example shown, the aromatase inhibitor Org 30958 (**8**) in Figure 5 (entry 3) the reduction of the double bond has to

[a] Since ^3H, ^{14}C and ^{35}S are weak β emitters, shielding of the radioactivity, *e.g.* by lead, is not necessary. Personal contamination by inhalation or ingestion is considered as a greater health risk. For this reason all chemical handlings are performed in well-ventilated fume cabinets located in restricted laboratories and volatile radioactive materials released are trapped immediately. Regular monitoring of personnel and equipment for radioactive contamination is essential.


①
Cl
$^3H_2$
Pd/C
KOH / EtOH
^3H
4
2
②
NIS
H^+
$^3H_2$
Pd/C
EtOH
6
7
5
③
$^3H_2$
Pd/C ; EtOAc
TsCl
Pyridine
TsO
$MeO^\ominus$
HS
Et–S–S–Et
9
10
11
12
13
8
④
CH_3COCl
^3H_2O
DME 40°C
14
14a
⑤
^3H_2O
$NaOCH_3$
DMF
LiC≡CH
15
15a
H^+
16
14b

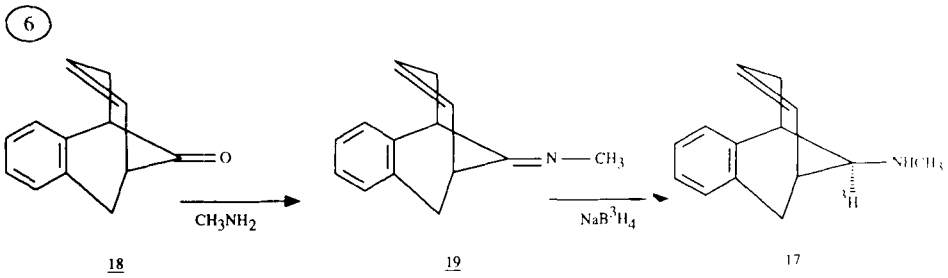


Figure 5. Examples of syntheses of tritiated compounds; 1: mianserin (**2**); *2: Org 4428* (**5**); *3: Org 30958* (**8**); *4: Org 3236* (**14**); *5: Org 6370* (**17**).

be done in an early stage of the synthesis since the other substituents at position 19 would be affected by the catalytic reduction. Labelling by exchange reaction with ^3H_2O are illustrated for the anti-conceptive steroid Org 3236 (**14**) (entry 4). This compound was tritiated either by reaction with $^3H_3O^+$ at positions 2, 4, 6 and 10 to give **14a** or at position 16 by exchange under alkaline conditions of the 17-oxo precursor followed by ethynylation to give **14b**. Since in the latter compound the tritium is incorporated into a stable position, this material could be applied for metabolism studies. Tritiated $NaBH_4$ can be used in the synthesis of tritiated alcohols[11], whereas imines can be reduced to obtained tritiated amines, as illustrated for the potential anti-epileptic drug Org 6370 (**17**) (entry 6).

The syntheses of ^{13}C-labelled compounds resemble normal laboratory organic syntheses with respect to scale and yields. For ^{14}C-labelled compounds, some examples of syntheses are given in Figure 6. For these materials (and this

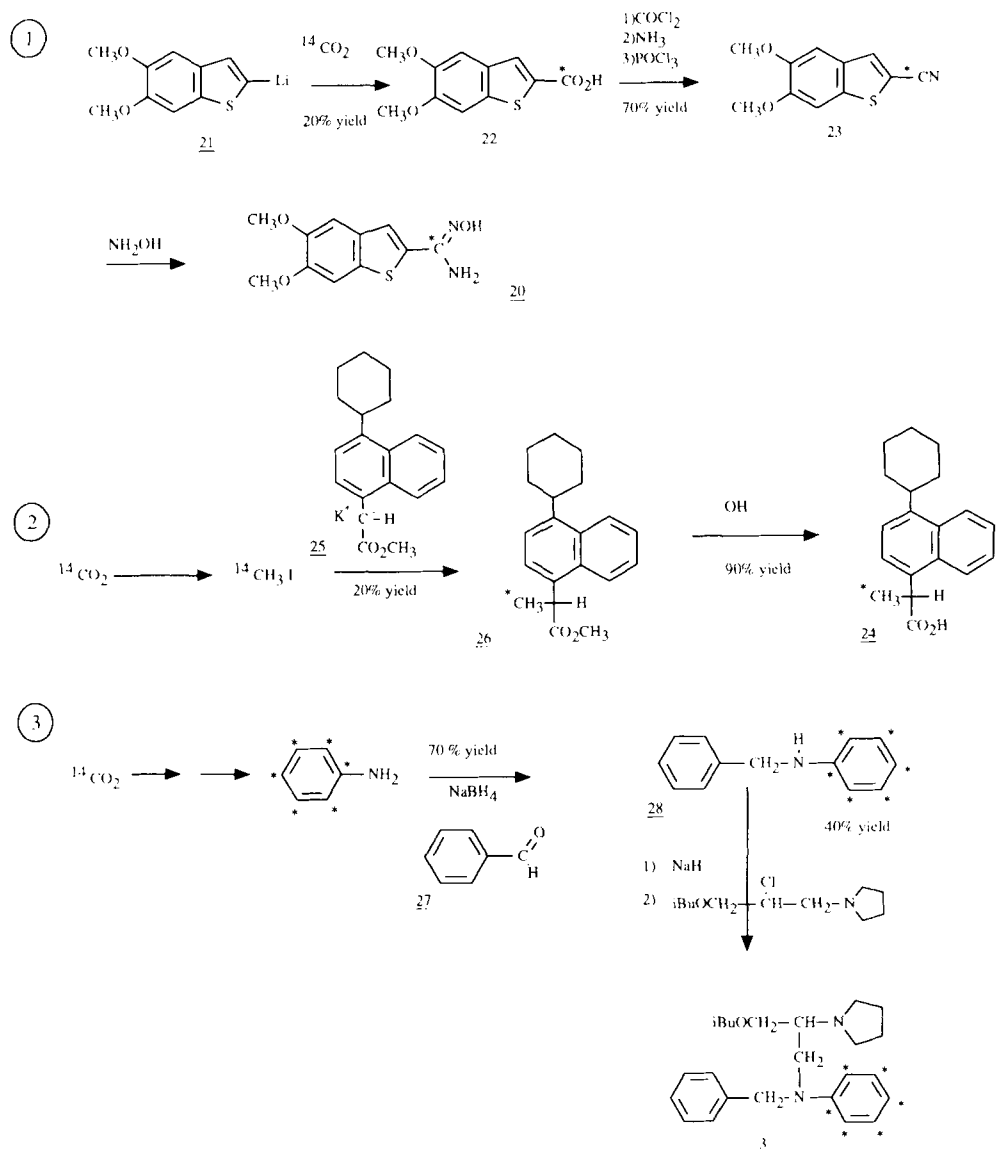


Figure 6. Examples of syntheses of ^{14}C-labelled drugs; 1: Org 30029 (**20**); *2: Org 7791* (**24**); *3: bepridil* (**3**).

holds also for ^{13}C) the limiting factors are the costs of the labelled reagents.

The cheapest starting material available is $^{14}CO_2$. Other simple low-molecular-weight compounds available from commercial suppliers (and prepared by them from $^{14}CO_2$) can also be used, as illustrated for the synthesis of [N-phenyl-^{14}C]bepridil (Figure 6, entry 3) where ^{14}C-aniline is used as starting material. The synthetic pathways and reaction conditions are selected in such a way that maximum radiochemical (instead of chemical) yields are obtained. For example, in the synthesis of the ^{14}C-Org 30029 (**20**; a compound active in the cardiovascular system, entry 1) we used excess benzothiophene for the carboxylation instead of excess CO_2. In the synthesis of ^{14}C-Org 7791 (**24**, an anti-inflammatory drug, entry 2) excess potassium salt **25** is used in the reaction with $^{14}CH_3I$. Because of the small scale on which the reactions are performed and the radioactivity involved the reaction conditions are less controllable and as a consequence radiochemical yields are in general lower than those obtainable in a non-radioactive synthesis.

Reaction products, even when formed in small yields, are easily detected because of their radioactivity: for instance,

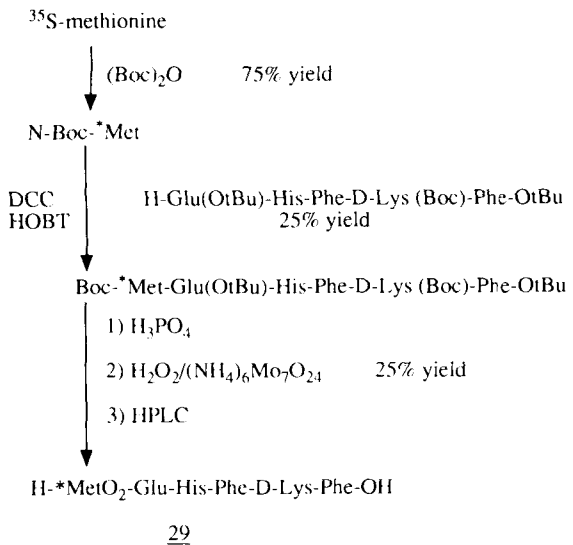


Figure 7. Synthesis of [^{35}S]Org 2766; BOC = tert-*butoxycarbonyl, HOBT = hydroxybenzotriazole.*

the carboxylation of the thiophene derivative **21** gives, in addition to desired 2-carboxylate, about 3% of the 3-carboxylate. In general, reaction of lithiated materials prepared by treatment of precursors with BuLi with $^{14}CO_2$ gives rise to the formation of addition products of butyl lithium with the Li carboxylate formed[12].

For ^{35}S-labelled materials, we have only experience with ^{35}S-labelled neuropeptides[13]. For the synthesis of these materials we start from the commercially available [^{35}S]methionine or [^{35}S]cysteine obtained from bacteria grown on [^{35}S]sulphate. Even with these low amounts (≈ 1 μg) normal peptide synthesis can be done provided that large excess of unlabelled peptide precursors are used as shown for the neuropeptide [^{35}S]Org 2766 (**29**) in Figure 7[14].

Radioactively labelled drugs are not stable as the isotopes decay, *e.g.* ^{14}C into ^{14}N and ^{35}S into ^{35}Cl. ^{3}H is special since it decays to ^{3}He resulting in the formation of a carbenium ion[15]. A typical example is shown in Figure 8: on storage of [13-^{3}H]mianserin as a solution in benzene for about 5 years, 13-phenylmianserin (**30**) was formed as a result of the decay process. More serious is the problem of radiolysis. As a consequence of the radioactive decay, reactive radicals, ions and molecules are formed, resulting in the chemical degradation or modification of the labelled drugs. For example, we have observed reduction of carbon–carbon triple bonds, N-oxide formation of aliphatic amines and degradation of peptides. The seriousness of this effect is, amongst other things, dependent on the specific activity of the material; while ^{14}C-labelled materials can be stored as such, tritiated and ^{35}S-labelled materials must be stored as dilute solutions, preferably in the presence of radical scavengers. Even under these conditions the compounds decompose slowly and, as shown in Figure 9, especially the half-life of the oxidation-sensitive mercapto compounds is limited to about a day.

Analytical characterisation

It is obvious that compounds that are labelled with non-radioactive isotopes and that are prepared in "large" amounts (> 100 mg) can be subjected to normal analytical procedures to assess identity and purity. Special features are the measurement of isotopic content and the localisation

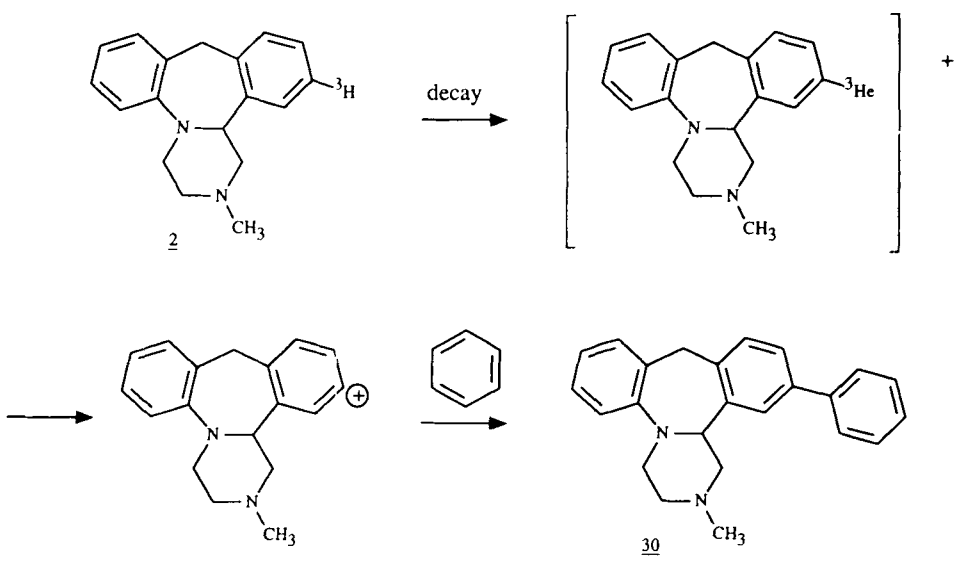


Figure 8. Formation of 13-phenylmianserin (**30**) *from [13-^{3}H]mianserin* (**2**).

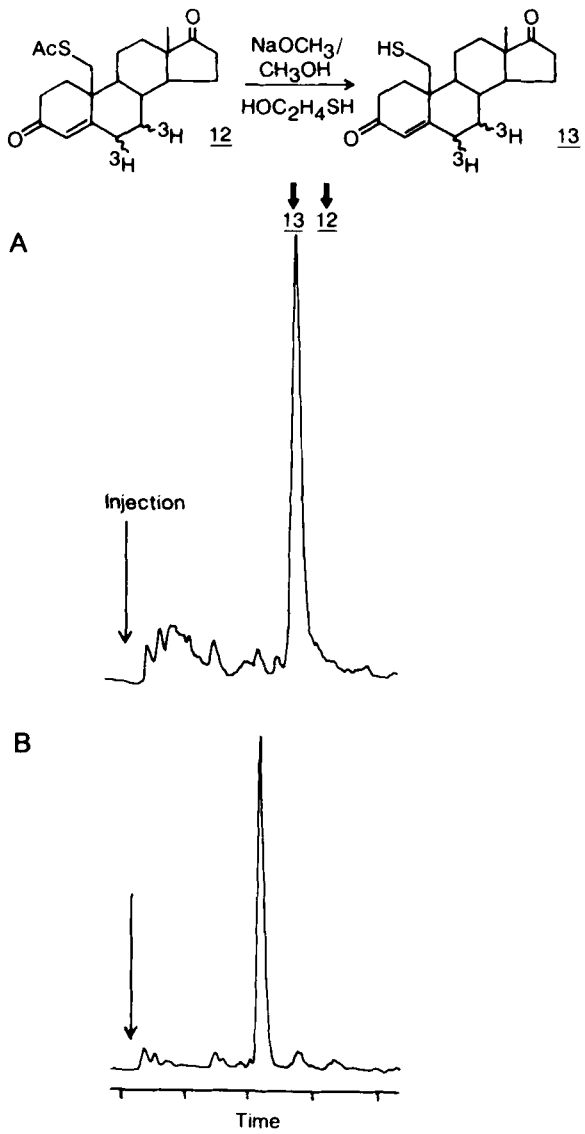


Figure 9. HPLC chromatograms of the reaction products formed on the hydrolysis of the S*-acetyl steroid* **12**.
A: 2.5 hours reaction; about 95% of S*-acetyl derivative* **12** *is converted into* **13**.
B: 48 hours reaction: about 90% of the SH derivative **13** *is converted into an unidentified oxidation product. Compound* **13** *could be converted into the stable (for at least 3 months) disulphide* **8** *by direct reaction with ethylsulphonyl thioethane (Figure 5, entry* ③*).*

of the label. Measurement of isotopic content is done by mass spectrometry, preferably using a soft-ionisation technique such as FD or CI. Determination of the position of the label in the molecules is established with NMR by observation of the isotope signals (^{13}C and ^{2}H NMR) or by changes in coupling patterns in the ^{1}H or ^{13}C spectra[16].
Since conclusions from biological and biochemical studies conducted with labelled materials are usually only based on the radioactivity measurements, quality control for this kind of material is even more important. The compounds are defined by their *chemical identity*, *radiochemical purity* (% of radioactivity present in the (correct) molecule) and *specific activity* (number of isotopes, expressed in Bq or Ci, present per molecule), while the *position of the label* should also be assessed. Depending on the isotope and thus the amount of material available, different techniques are applied. These are summarized in Table I.
Radiochemical purities are determined by chromatography combined with radioactivity detection. The specific activity is determined by mass spectrometry using soft-ionisation techniques; in case of tritiated material chromatographic separation is preferably included (GC/MS of LC-continuous-flow FAB) to circumvent interference by scavengers and impurities in the solvents used for storage of the material. A typical example is given in Figure 10. For ^{35}S material more sensitive techniques such as HPLC assays have to be applied.
Especially for tritiated and ^{35}S-labelled drugs, the specific activities of the products are not necessarily the same as those of the starting material of reagents used for the synthesis of these compounds. For reductive dehalogenations and reductions with $^{3}H_2$ catalysed by metals, exchange of the $^{3}H_2$ with ^{1}H from solvents is a common phenomenon. For tritiated and ^{35}S-labelled drugs, which are both purified by HPLC, dilution can occur by using the unlabelled drug to calibrate and inactivate the HPLC (memory effect). For ^{3}H-labelled compounds, this effect on the special activity is only 10–30% but for the [^{35}S]methionine peptides described earlier this dilution is more substantial and can result in a specific activity of 10–15% of the theoretical maximum[14].
The position of the labels is determined by NMR. ^{3}H-labelled drugs can be measured directly because ^{3}H is a NMR-active isotope (nuclear spin $\frac{1}{2}$; resonance frequency 1.06664 and sensitivity 1.21, the latter two in comparison with ^{1}H). ^{3}H NMR of organic material was first published in 1964 by *Tiers* et al.[17] and it has developed into a powerful technique for the analysis of tritiated material.
The quality of a ^{3}H NMR spectrum is determined by the amount of material that can be analysed. This quantity is limited by the availability of material, safety limits and, more important, by the possible interference of radiolysis. The ^{3}H NMR coil dimension and the double NMR tube used because of safety, limit the actual volume to 0.1 ml and for a typical 5 mCi (185 MBq) sample this means a radiation dose of $7 \cdot 10^{11}$ keV/ml/min. To reduce the possible decomposition reactions, NMR samples are always diluted with unlabelled "carriers" and after recording of the ^{3}H spectrum a ^{1}H spectrum is recorded to monitor any possible decomposition.

Table I Methods for characterization of radioactive drugs.

	Isotope		
	^{3}H	^{14}C	^{35}S
identification	chromatography/MS	chromatography MS/^{1}H NMR	chromatography
radiochemical purity	chromatography/^{3}H NMR	chromatography	chromatography
specific activity	chromatography/MS	MS	chromatography
position of label	^{3}H NMR	^{13}C NMR/MS (EI)	

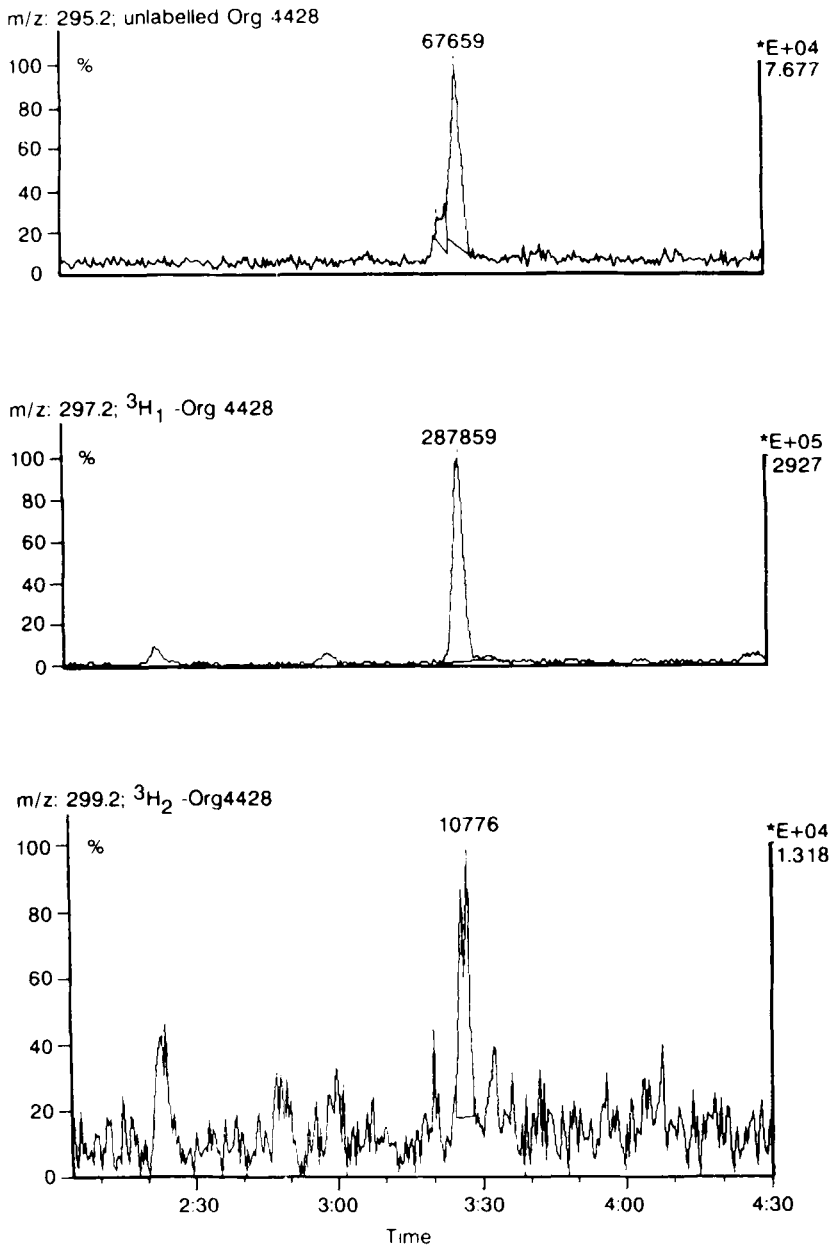


Figure 10. GC/MS pattern of tritiated Org 4428 (**5**); *column: DB-1 with the detection EI; labelling pattern: 18.8%* $^3H_0$; *80.8%* $^3H_1$; *0.4%* $^3H_2$.

In Figure 11, an example of a good-quality ^3H NMR spectrum is given for tritiated Org 4428 (**5**) synthesized as described earlier. In this spectrum even the ^{13}C satellites are visible, *i.e.* the detection limit for this particular amount (8 mCi = 300 MBq; 100 μg) is about 0.5%. With more complicated spectra, such as the ^1H-coupled spectrum, or with less material this detection limit increases to 3–5%.

In addition to its applications for identification and measurement of the radiochemical purity of tritiated material, ^3H NMR is mainly used, as shown in Figure 12 and 13, for determination of the localisation and distribution of the label and measurement of the different mono- di- or multi-tritiated species. The main problem with tritiation reactions are unexpected exchange reactions. In the ^1H-decoupled spectrum of Org 4428 (**5**) we see the expected signal for position 12 and also some tritium at position 6. Since the starting iodinated material did not contain any isomeric iodo–derivative or diiodo–material, labelling at position 6 is probably caused by acidic exchange with ^3HI formed during reductive deiodination.

In Figure 12 the spectrum of the sulphur-containing steroid Org 30958 (**8**) is given. From the tritium spectrum it can be concluded that 75% is present as [7β-^3H]Org 30958, 12.5% as [6β, 7β-$^3H_2$]Org 30958 (incomplete detritiation of position 6β) and 12.5% as [6α,7β-$^3H_2$]Org 30958 (position 6α labelled through isomerisation of 6β). For the ditritiated material, $^3H-^3H$ couplings, which are 14% larger than the corresponding $^1H-^1H$ couplings, are present.

As expected, exchange-labelling procedures with $^3H_2$ or ^3H_2O (and catalysed by acid/base or metals) result in even more complex distributions of labels. This is illustrated in Figure 13 for the steroid Org 3236 (**14**). All "enolisable" positions 2α, 2β, 4, 6α, 6β and 10 are more or less equally labelled. This spectrum clearly demonstrates the isotopic

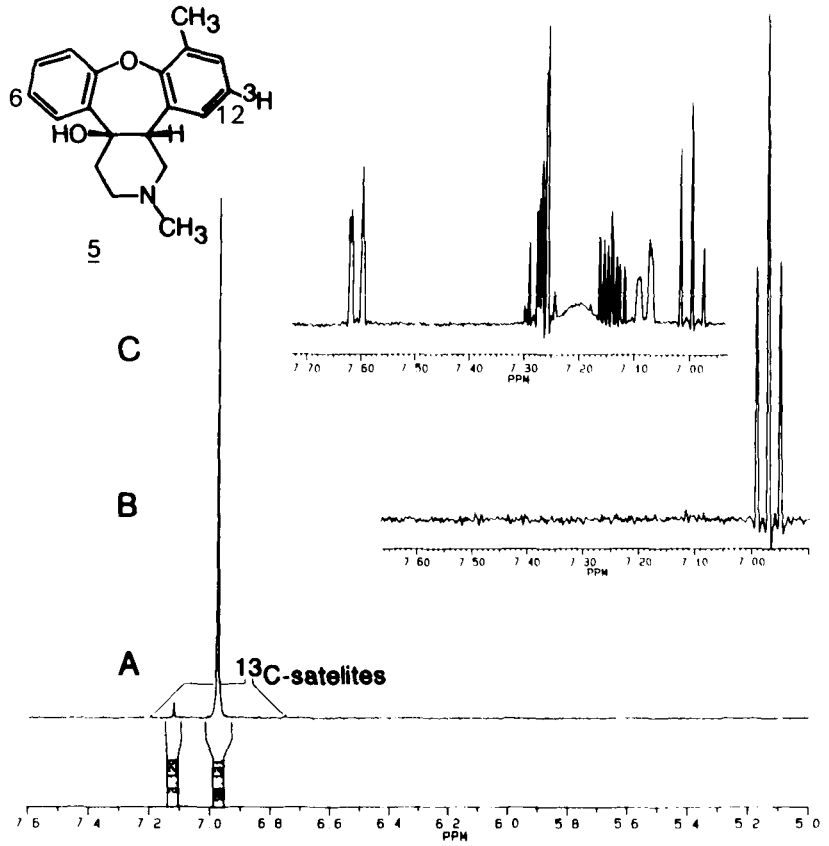


Figure 11. Aromatic part of NMR spectra (in C^2HCl_3) of [^{3}H]Org 4428 (**5**); *A: ^{1}H-decoupled ^{3}H NMR spectrum; B: ^{1}H-coupled ^{3}H NMR spectrum; C: ^{1}H NMR spectrum.*

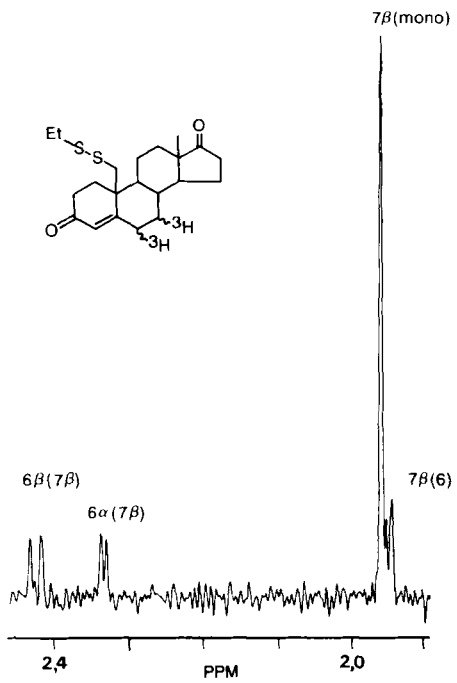


Figure 12. ^{3}H NMR spectrum [in $(C^2H_3)_2SO$] of Org 30958 (**8**); *^{1}H-decoupled.*

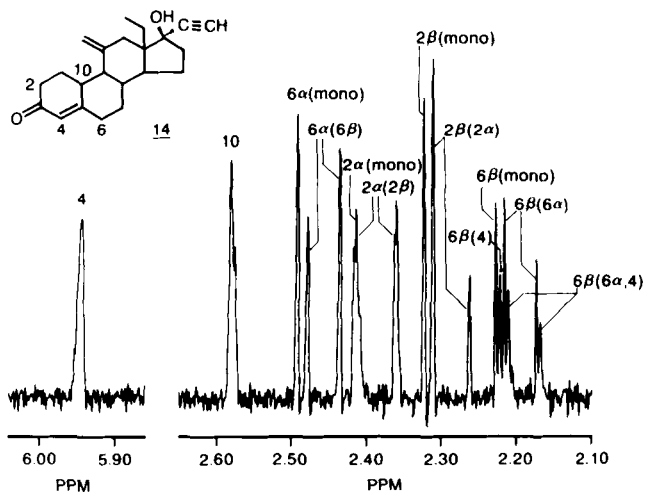


Figure 13. ^{3}H NMR spectrum (in C^2HCl_3) of [^{3}H]Org 3236 (**14**); *^{1}H-decoupled.*

shift of about 0.02 ppm for geminally ditritiated material; for vicinally ditritiated material this isotope shift is about 0.01 ppm.

For ^{14}C-labelled drugs one can use the small isotope shift in ^{1}H NMR caused by ^{14}C substitution (– 3.5 ppb)[19]. One can also use the absence or reduction of the ^{13}C NMR signal of the labelled position as shown in Figure 14.

Concluding remarks

Isotopically labelled drugs play a major role in drug development. Their applications, synthesis and characterization varies widely with the various isotopes involved.

Even with the development of more sensitive bio-analytical methods, isotopically labelled drugs will remain essential

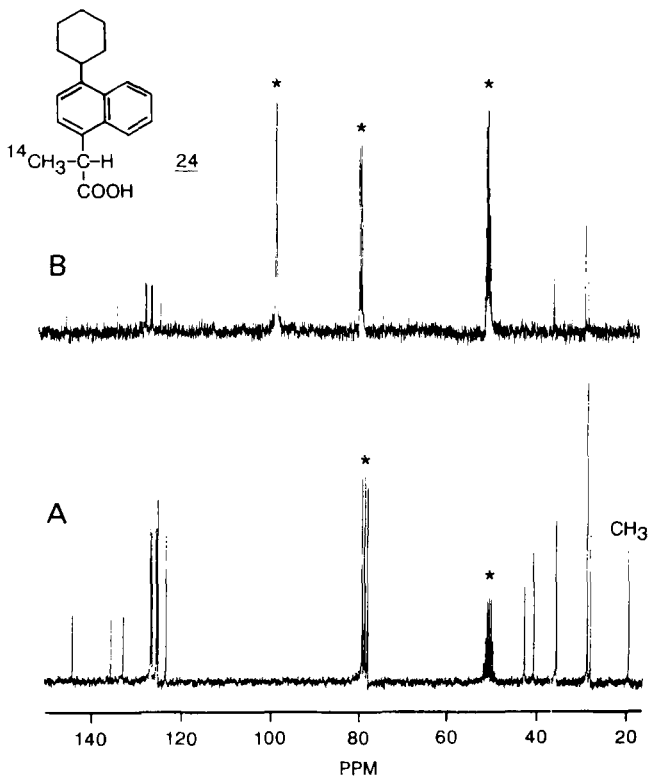


Figure 14. ^{13}C *NMR spectrum in* $C^2HCl_3/C^2H_3O^2H$ *of Org 7791* (**24**); *broad band decoupled. A: unlabelled material; B:* $[\alpha\text{-}^{14}CH_3]$*-labelled material; * NMR solvents.*

for the assessment of drugs in biological systems, especially for metabolism and excretion studies. A new approach is the use of ^{11}C-labelled materials; because this a β^+-emitter with a short half life ($t_{\frac{1}{2}}$ 20 min), it is possible to measure non-invasively the concentration of the *label* in living systems and it can be used for special mechanism of action studies *e.g.* uptake of the drug in brain[20].

Acknowledgements

The authors thank their colleagues in the Departments of Drug Metabolism and Kinetics and Analytical Chemistry for their contributions to the investigations cited in this paper.

References

[1] *T. A. Baillie*, Pharmacol. Rev. **33**, 81 (1981).
[2] *J. M. Miles* and *M. D. Jenson*, J. Parenter. Enternal. Nutr. **15**, 908 (1990).
[3] *J. S. Rohrer* and *J. D. Olechno*, Anal. Chem. **64**, 914 (1992).
[4] *F. M. Kaspersen, J. v. Acquoy, G. L. M. v. d. Laar, G. N. Wagenaars* and *C. W. Funke*, Recl. Trav. Chim. Pays-Bas **103**, 32 (1984).
[5] *F. M. Kaspersen, C. W. Funke, E. M. G. Sperling, F. A. M. v. Rooy* and *G. N. Wagenaars*, J. Chem. Soc., Perkin Trans II, 585 (1985).
[6] *J. Vink*, Mass Spectrum Rev. **1**, 349 (1982).
[7a] *W. N. Wu, J. F. Hills, S. Y. Chang* and *K. T. Ng*, Drug Metab. Dispos. **16**, 69 (1988);
[7b] *L. P. C. Delbressine*, unpublished results.
[8] *E. Buncel* and *J. R. Jones* (eds): Isotopes in the Physical and Biomedical Sciences, Volume I, Labelled Compounds (Part A) Elsevier, 1987.
[9a] *H. Andres, H. Morimoto* and *P. G. Williams*, J. Chem. Soc. Chem. Commun. 628 (1990);
[9b] *H. Andres, H. Morimoto* and *P. G. Williams* in Synthesis and Application of Isotopically Labelled Compounds 1991, 40, *E. Buncel* and *G. W. Kabalka* (eds.), Elsevier 1992.
[10] *P. Konradsson, D. R. Motoo, R. E. McDevitt* and *B. Fraser-Reid*, J. Chem. Soc. Chem. Commun. 270 (1990).
[11] *F. M. Kaspersen, I. H. G. Schlachter* and *G. W. K. van Dedem*, J. Labelled Comp. Radiopharm. **29**, 241 (1991).
[12] *F. M. Kaspersen, F. A. M. v. Rooy, E. M. G. Sperling* and *J. H. Wieringa*, J. Labelled Comp. Radiopharm. **27**, 1055 (1989).
[13] *J. W. v. Nispen, J. A. J. Hannink, F. M. Kaspersen* and *E. M. G. Sperling*, Peptides, 267 (1986).
[14] *F. M. Kaspersen, J. W. van Nispen, E. M. G. Sperling* and *J. F. Vader*, J. Labelled Comp. Radiopharm., accepted for publication.
[15] *F. Cacace*, Science **250**, 392 (1990).
[16] *E. M. M. van den Berg, F. J. H. M. Jansen, A. T. J. W. de Goede, A. U. Baldew* and *J. Lugtenburg*, Recl. Trav. Chim. Pays-Bas **109**, 287 (1990).
[17] *G. V. D. Tiers, C. A. Brown, R. A. Jackson* and *T. N. Lahr*, J. Am. Chem. Soc. **86**, 2526 (1964).
[18] *E. A. Evans, D. C. Warrell, J. A. Elvidge* and *J. R. Jones*, Handbook of Tritium NMR Spectroscopy and Applications, John Wiley and Sons, 1985.
[19] *C. W. Funke, F. M. Kaspersen, E. M. G. Sperling* and *G. N. Wagenaars*, J. Chem. Soc. Chem. Commun. 462 (1986).
[20] *J. W. v. Nispen, W. P. A. Janssen, P. A. T. A. Melgers, P. S. L. Janssen, J. F. G. A. Jansen* and *W. Vaalburg*, Int. J. Pept. Protein Res. **36**, 167 (1990).
[21] *A. P. A. de Man, L. P. C. Delbressine* and *F. M. Kaspersen*, unpublished results.
[22] *H. J. M. v. Hal, J. E. Paanakker, E. M. G. Sperling* and *F. M. Kaspersen*, to be published.