Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 19 January 2010, Revised 10 March 2010, Accepted 29 March 2010 Published online 30 July 2010 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.1792

Labeling of PKC412 – a fermentative radiolabeling

P. Burtscher,[a] H. Haecker,[b] and T. Moenius[a]*

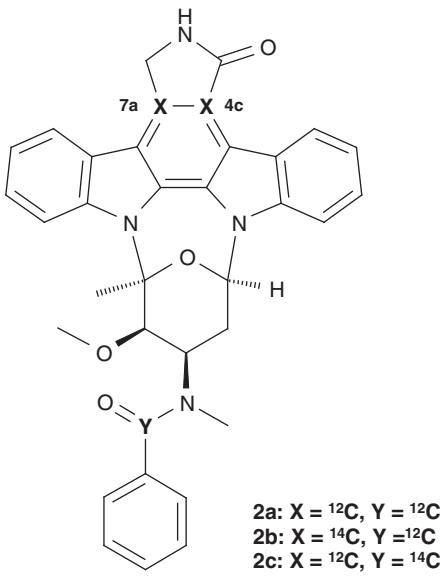
Addition of [3-^{14}C]D/L-tryptophan to the fermentation broth allowed radiofermentative labeling of staurosporine. The approach providing [4c,7a-$^{14}C_2$]staurosporine in a range from 700 to 1300 MBq/mmol was straightforward and easy to handle as well as safe from a radioprotection point of view. The presented example underlines that close cooperation of microbiologists and radiochemists is a requisite for the success of this approach.

Keywords: C-14 labeling; fermentation; radiofermentation; staurosporine; PKC412

Introduction

PKC412 (*N*-Benzoylstaurosporine) **2a** is a indolocarbazole-type multikinase inhibitor with potential anti-angiogenic and anti-neoplastic activities. Synthetically, it is a *N*-benzoyl derivative of fermentatively produced staurosporine **1a** (Figure 1).

To support the registration, C-14 labeled PKC412 **2b** was requested for environmental risk assessment studies. Previous ADME studies were performed with *N*-benzoyl-[carboxyl-^{14}C]-staurosporine **2c**.[1] Despite its metabolic stability in ADME experiments, labeling of the skeleton of staurosporine moiety was requested for this type of study. Strategies such as total synthesis or reconstitution appeared to be too ambitious and time-consuming. Therefore, adaption of the fermentation process by addition of C-14 labeled precursors was considered to be the method of choice.



2a: X = ^{12}C, Y = ^{12}C
2b: X = ^{14}C, Y = ^{12}C
2c: X = ^{12}C, Y = ^{14}C

Figure 1. Structure of PKC412.

Microbiological methodologies do not belong to the daily instrument of radiochemists and therefore require close cooperation with the corresponding experts. Our approach was strictly product-driven and does therefore not address all methodological issues of a fermentation development. Nevertheless, because this method is not common and proved to be a straightforward labeling strategy for a complex molecule, this example is considered of interest for radiochemists.

Fermentative labeling

Fermentative radiolabeling has recently been reviewed.[2] In case of structurally highly complex molecules, this approach demonstrated superiority over synthetic approaches. Convincing examples are Acarbose,[3] Avermectin,[4] Geldanamycin[5] and Rapamycin.[6] As summarized, the fermentation process is strongly influenced by experimental parameters such as the producing organism, the growth medium, the type of precursor added, the process conditions (i.e. temperature, concentration of the precursor, time points of addition and harvest) and the radiosensitivity of the organism. Their impact has to be investigated and optimized.

In the case of staurosporine, some data are available from literature. Cordell investigated the biosynthesis of staurosporine identifying L-Trp,[7] D-glucose[8] and *S*-methylmethionine[9] as possible precursors. Tracer experiments indicated[7] that the indolocarbazole moiety is derived from two intact tryptophan molecules. Therefore, L-tryptophan appeared to be a suitable precursor. The same communication, however, provides evidence that increased concentrations of L-tryptophan might suppress the staurosporine production. Subject of further

[a]*Novartis Pharma Ltd., DMBA-IL(EU), CH-4002 Basle, Hueninger Street, Switzerland*

[b]*Sandoz Kundl, Biochemiestrasse 20, A-6250 Kundl, Austria*

**Correspondence to: T. Moenius, Novartis Pharma Ltd., DMBA-IL(EU), CH-4002 Basle, Hueninger Street, Switzerland.*
E-mail: thomas.moenius@novartis.com

optimization was balancing the incorporation with the specific incorporation.

Adaption of the fermentation process

SANDOZ Kundl provided us with a *Streptomyces longisporoflavus* high-producing strain and adapted the existing fermentation protocol to our needs. Without definite optimization, the impact of the amount of precursor added, of the time point of feeding and harvesting were investigated.

Attempts to modify the added precursor to L-Trp-OMe[10] in order to prevent inhibition of the system were not successful. Therefore, L-Trp appeared to be the building block of choice. First experiments with [$^{13}C_6$]L-Trp indicated efficient incorporation (>50%, determined by ^{13}C-NMR) at a level of 1 g/kg. Taking into consideration that two L-Trp moieties contribute to one molecule of staurosporine provided evidence that the method has a distinct chance of realization.

Adaption to the radiofermentation

In contrast to the pre-experiments, a racemic mixture of D/L-Trp[11] was used for the radiofermentation. Owing to its commercial availability synthetically produced [3-^{14}C]D/L-Trp was selected as building block resulting in the labeling of the positions 4c and 7a of the staurosporine moiety.

Owing to differences in the fermentation equipment, the transfer of the protocol to the isotope lab revealed some difficulties. Lower shaking intensity (due to lower extension) and no capability to control the humidity of our shaking device resulted in a generally lower titer and a considerably higher inter-experimental variability. Despite experimental attempts to compensate for these effects (i.e. by increasing the shaking velocity and by saturation of the circulating air stream with water at 35°C), the quality of the above experimental data could not be reached.[12]

Orientating experiments under these conditions gave evidence that an increasing addition of D/L-Trp (15, 30 and 60 mg/50 ml) raised the incorporation significantly. Addition of 30 mg D/L-[^{13}C]Trp (=15 mg L-Trp)/50 ml produced about 30% of doubly labeled staurosporine. Based on the specific activity of the [3-^{14}C]D/L-Trp (50 mCi/mmol) employed, this would be sufficient to meet the customer's requirement (20 mCi/mmol). Although significantly less productive, up to 50% of the potential appeared to be sufficient and prompted us for a first radiolabeled run.

The goals of the first radiolabeled run were to verify the above range of data, to test the organism for radiosensitivity and to determine the amount of volatile activity (i.e. [^{14}C]CO_2] produced during the fermentation process. For this reason, the air was continuously circulated through 1 N aqueous NaOH to capture any [^{14}C]CO_2.

First run with radiolabeled precursor

Surprisingly, this run (entry 1) showed very high yields of staurosporine providing no indication of radiation sensitivity at the applied radio concentration. The radiochemical purity of the crude material was >90% resulting in 78 MBq of **1b** after HPLC-purification. Even when the radiochemical yield amounted to only 10%, only 4% of the total radioactivity was found in the aq. NaOH impinger solution giving evidence for low volatile activity. Lower than expected from experiments with C-13 was the specific activity at 795 MBq/mmol. This number was close to the lower limit of the requested range (20–40 mCi/mmol). But as the titer was unusually high, this specific activity was considered to be minimal. It is interesting to note that the D-[^{14}C]Trp could (partially) be recovered during work-up.

Second run with radiolabeled precursor

Although not robust, these results confirmed the general applicability of the process and underlined its suitability for the production of C-14-labeled staurosporine. A repetition (30 mg/50 ml) on a preparative scale (i.e. using more fermentation flasks) appeared to be reasonable. Entries 2 and 3 were performed in the same run. In order to minimize the risk of a too low specific activity, the addition was raised to 40 mg/50 ml for five flasks. In this run (entries 2 and 3), the titer was 50% lower. The specific activities, however, were significantly higher. Under identical reaction conditions raising the addition from 30 mg/50 ml to 40 mg/50 ml increased the average specific activity from 1000 to 1397 MBq/mmol. In contrast to entry 1, the radiochemical purity of the crude material was less (74–78%) resulting in 123 and 141 MBq after HPLC-purification, respectively, correlating to a radiochemical yield of 7.6 and 6.5%. In this run, the volatile activity amounted to 2% of the total activity employed. Finally, 4520 MBq of [3-^{14}C]D/L-Trp provided 342 MBq of [4c,7a-$^{14}C_2$]staurosporine **1b**.

Formation of [$^{14}C_2$]PKC412

In a final synthetic step, the [4c,7a-$^{14}C_2$]staurosporine **1b** was *N*-benzoylated to obtain [$^{14}C_2$]PKC412 **2b** (Figure 2).

Experimental

Materials

Strain: *Streptomyces longisporoflavus* GLS7 high-producing mutant of *Streptomyces longisporoflavus R19*.[13]

Procedure

(a)*Pre-culture* medium containing sucrose and soybean flour, pH adjusted to 6.8–7.5, standard sterilization conditions. Inoculated with 2–6% frozen culture, cultivation time 48–56 h.
(b)*Fermentation broth* containing glucose, sucrose, soybean flour, ammonium sulfate calcium sulfate and some antifoam. pH adjusted to 6.8–7.5.
(c)*Fermentation conditions*
Inoculated with 10% seed culture.
Addition of precursor after 24 h, amount of precursor added (see Table 1), cultivation time 140–150 h.
(d)*Work-up*: The combined phases basified with 6 M NaOH to pH 9.2 were combined with ethyl acetate and filtered over Super Cel. The organic phases contained the crude C-14 labeled staurosporine, which was purified by using flash chromatography.

The identity and radiochemical purity were determined by using HPLC, MS and ^{1}H-NMR. The identification of the labeling position by depletion of the corresponding ^{13}C-NMR was not possible due to the insufficient signal intensity.

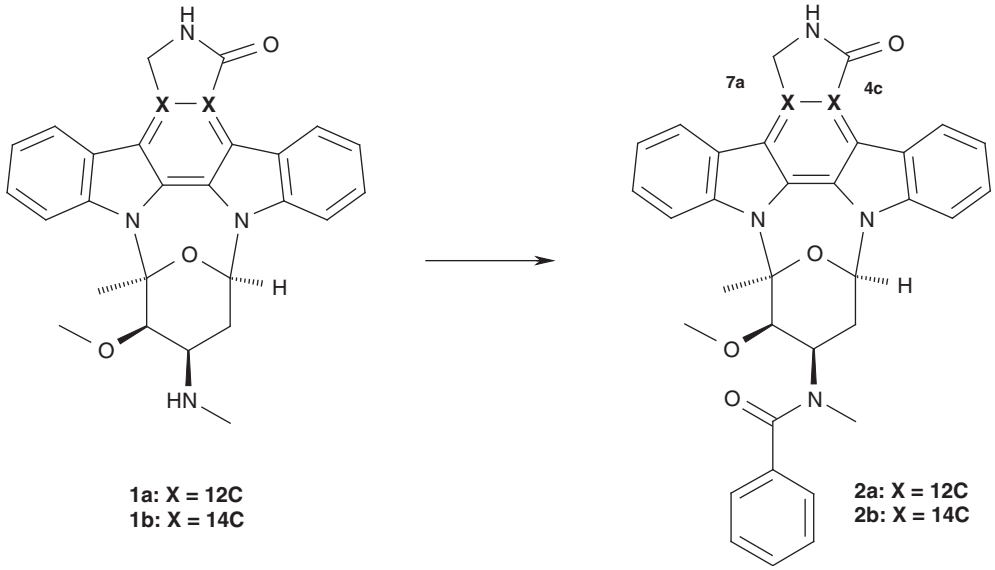


Figure 2. Synthesis of [^{14}C]PKC412 **2b** from [4c,7a-$^{14}C_2$]staurosporine **1b**. Reaction conditions: (1) benzoic acid, 2-chloro-1-methyl pyridinium iodide, triethylamine, CH_2Cl_2 12 h, r.t, flash chromatography.

Table 1. Radiofermentative synthesis of [^{14}C]Staurosporine **1b** under addition of [3-^{14}C]D/L-Trp

	Activity employed (MBq)	Flasks ♯	Volume (ml)	Conc. (mg/ml)	Activity NaOH (MBq)	Activity water (MBq)	Activity crude (MBq)	Purity RA, crude (%)	Activity purified (MBq)	Specific activity (MBq/mmol)	Purity RA (%]	Yield (%]
1	740	2	50	30/50	25	200	100	90	78	795	98.5	10.5
2[a]	1620	5	50	30/50	[b]	565	n.d.	78	123	1002	98.6	7.6
3[a]	2160	5	50	40/50	[b]	810	n.d.	74	141	1397	97.6	6.5

n.d. not determined.
[a]Entries 2 and 3 were processed in the same run.
[b]The combined amount of volatile activity was 79 MBq.

Acknowledgements

We thank Dr. A. Glänzel and R. Lehmann for their valuable support.

References

[1] I. Rodriguez, unpublished results.
[2] R. Voges, J. Heys, T. Moenius, *Preparation of Compounds Labeled with Tritium and Carbon-14*, Wiley, New York, **2009**, 618–634.
[3] a) W. Maul, L. Müller, J. Pfitzner, E. Rauenbusch, H. M. Schutt, *Drug Res.* **1989**, *39*, 1251–1253; b) D. Seidel, H. Wehlmann, J. Lenz, *J. Label. Compd. Radiopharm.* **2007**, *50*, 933–943.
[4] M. A. Wallace, D. C. Dean, R. L. Ellesworth, T. Marks, D. G. Mellilo, P. Masurekar, in *Synthesis and Applications of Isotopically Labelled Compounds 1994* (Eds.: J. K. Allen, R. Voges), Wiley, Chicester, **1995**, 605–612.
[5] G. F. Taylor, K. K. Foarde, T. D. Webber, J. A. Kepler, *J. Label. Compd. Radiopharm.* **2001**, *44*, 165–168.
[6] Th. Moenius, R. Voges, M. Mahnke, P. Burtscher, Y. Metz, Ch. Guenat, *J. Label. Compd. Radiopharm.* **1999**, *42*, 29–41.
[7] a) D. Meksuriyen, A. Cordell, *J. Nat. Prod.* **1988**, *51*, 893–899; b) S-W. Yang, G. A. Cordell, *J. Nat. Prod.* **1997**, *60*, 788–790.
[8] S-W. Yang, G. A. Cordell, *J. Nat. Prod.* **1996**, *59*, 828–833.
[9] L.-J. Lin, G. A. Cordell, unpublished results, **1992**.
[10] Z. H. Ahmed, R. R. Fiala, M. W. Bullock, *J. Antibiot.* **1993**; *46*: 614–622.
[11] Based on reference 7a no impact is expected.
[12] It is therefore recommended to adapt the instrumentation of the isotope laboratory to that of the microbiological department.
[13] Y. Cai, A. Fredenhagen, P. Hug, H. Peter, *J. Antibiot.* **1995**, *48*, 143–148.