Technology-driven isotopic labelling in API development

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Isotopic labelling of modern active pharmaceutical ingredients (APIs), particularly of complex targeted therapies, is becoming more challenging, and material shortages compound the issue for carbon-14. The synthetic design for an isotopic analogue depends on a number of interdependent factors that must be carefully considered, along with any prior knowledge of the molecule, to ensure that a metabolically stable labelling position is achieved. The selection of appropriate labelled starting materials is limited, and the potential savings from beginning a synthesis with simple materials must be carefully weighed against the increased labour cost of a longer synthesis. The application of biocatalytic tools, for example, can introduce complex functionality in a single step and significantly help reduce the cost of labelled biomolecules and chiral compounds. Almac's Isotope Chemistry group has benefited from amalgamation with Biocatalysis and interacts with the company's Physical Sciences and Peptide Protein Technologies groups.

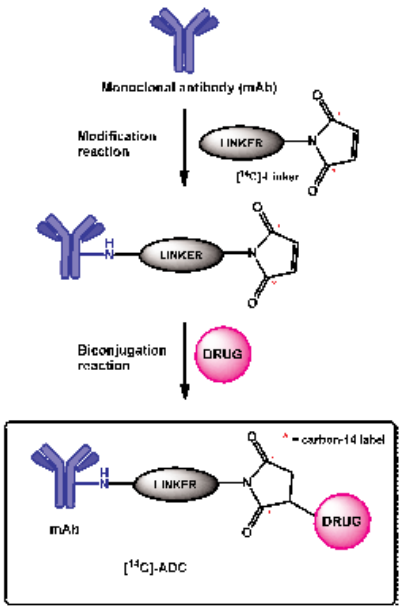

Fig 1. Carbon-14 labelling of an ADC.

Integration with other technologies

Almac actively integrates isotope chemistry with other in-house technologies, with the aim of finding fit-for-purpose solutions beyond the traditional realms of chemical synthesis. This often gives Almac new and attractive synthesis options and shorter synthetic routes. Furthermore, this integration ensures that best-in-class technical solutions can be found to the many challenges that contract isotope chemistry brings. This integration platform is best illustrated in the areas of bioconjugation, isotopically-labelled peptides and small molecules through to biocatalysis covering biotransformations and fermentation processes. All of these can be carried out in the regulatory environment of cGMP and investigated medicinal product (IMP) manufacture. Typical projects are highlighted within this article.

Bioconjugation products

Almac's radiochemists have successfully completed multiple projects involving carbon-14 labelling of antibody drug conjugates (ADCs). The strategy for isotopic labelling of the ADC is to prepare a product with the label in either the linker or in the payload or in both units. An example prepared at Almac is shown in Fig 1. The radiolabelling of this ADC was achieved via the carbon-14 labelled linker. The ester moiety of the [14C]-linker reacts with a random surface epsilon-amino group of lysine on the antibody to form a stable amide bond in the [14C]-linker-modified antibody. The terminal [14C]-maleimide moiety on the linker is then used in the conjugation reaction with the cytotoxic payload.

This produces a non-reducible thioether linker and the ADC contains two carbon-14 labels allowing for a maximum specific activity of 120 mCi/mmol per drug molecule. The carbon-14 labelled ADC then undergoes a purification process using ultrafiltration and diafiltration. A critical function of this system is to remove unbound [14C]-linker and/or cytotoxic drug to ease purification using hydrophobic interaction chromatography (HIC). This enables the isolation of carbon-14 labelled ADC, containing no labile cytotoxic drug.

Peptide products

In some instances, the customer will require the synthesis of modified carbon-14 peptides through the modification of carbohydrates, polysaccharides, and glycoconjugates, including PEGylated materials, BIOTINylation, biopolymers, or cytotoxic and highly potent compounds (Fig 2). The PEGlyation of peptides can be challenging, and these PEG covalent modifications require a reactive or targetable functional group at one end of the carbon-14 peptide. The simplest method to PEGylate carbon-14 peptides - which have a primary amine linker - is to use a PEG compound that contains an activated ester group at one end. An example includes the preparation of a biotinylated carbon-14 containing an 84 amino acid sequence. In this target, the unlabelled 83-mer resin-bound peptide was first synthesised using the SPPS approach. The terminal Fmoc amino acid protecting group was cleaved and the carbon-14 label introduced via N-Boc-L-[U-14C]isoleucine. Cleavage of the protecting group followed by biotinylation, N-Boc cleavage produced the 84-mer carbon-14 labelled peptide. Resin cleavage released the [14C]peptide-Biotin, which was purified and lyophilised, giving product with a radiochemical purity (HPLC) >98 area%, chemical purity (HPLC) >98 area% and specific activity >300 mCi/mmol.

Application of nitrilase

Nitrilase enzymes have been applied to the conversion of carbon-14 labelled nitrile groups through to the corresponding radiolabelled carboxylic acid. A rapid screen of nitrilase enzymes was carried out to identify the most effective enzyme which was used to produce the desired labelled product in good yield and excellent purity. The selected enzyme was

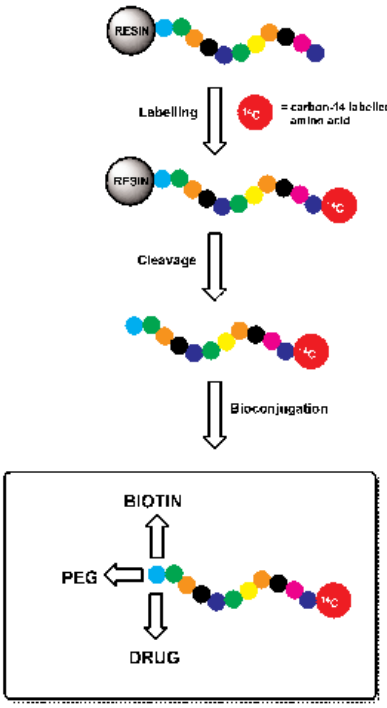

Fig 2. Synthesis of functionalised carbon-14 peptides.

then used under neutral conditions, thereby stopping hydrolysis of other sensitive moieties (Fig 3). Interestingly, all traditional synthetic alternatives failed to give the same intermediate in acceptable purity.

Application of P_{450} enzymes

The increased demand for oxidative metabolite synthesis has resulted in more and more research and published literature in the area of P_{450} enzymes. Their utilisation in practical synthesis of Phase 1 metabolites has led to a greater diversity of off-the-shelf catalysts that can be used by the synthetic chemist. The ease of access of P_{450} enzymes, even from published gene sequences, meant Almac was able to complete a carbon-13 project where the customer required the corresponding carbon-13 metabolite (Fig 4). Almac simply identified the P_{450} gene sequence from the literature and obtained the synthetic gene. The gene was cloned and expressed and the isolated enzyme obtained from fermentation was used in the isotope labs to easily access the customer metabolite incorporating the desired carbon-13 labelled sites.

Application of carbonyl reductase enzymes (CREDs)

Carbonyl reductase (CRED) enzymes have been used extensively in the preparation of chiral alcohols at scale and are now being used to incorporate deuterium into the API backbone. A recent project involved CRED screening to identify a CRED that was able to

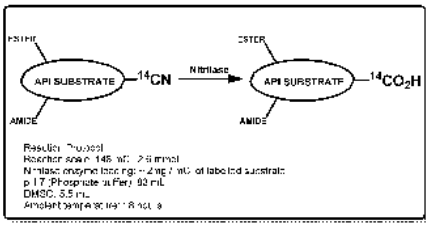

Fig 3. Nitrilase-mediated hydrolysis of a carbon-14 nitrile moiety to carbon-14 carboxylic acid.

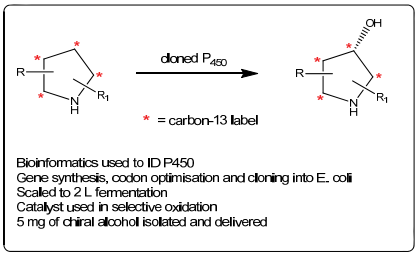

Fig 4. P450-mediated hydroxylation of a carbon-13 API.

form the chiral alcohol with >99.5% conversion and >99.2% ee. Key to the process was the use of deuterium-labelled glucose, which was consumed with glucose dehydrogenase (GDH) to turn over the NADP cofactor in the CRED reduction of the ketone to yield the chiral labelled alcohol. The product was isolated after a conventional solvent aqueous work-up to yield the product in 93% wt/wt yield and with >99.5% ee.

Fermentation products

Whole cell fermentation is an attractive approach to access complex natural products. Almac is using its fermentation methodology as a cost-effective method over traditional chemical synthesis to enable carbon-14 labelling of numerous biologically active complex molecules to be utilised in metabolism, toxicology and biodistribution studies. Almac designs and carries out custom fermentation to maximise the specific activity of the final product and provide labels in the biomolecule.

For example, the biosynthesis of polyketides involves the progressive addition of six (2S)-methylmalonyl-Coenzyme A extender units to one propionyl-CoA starter unit. (2S)-Methylmalonyl-Coenzyme A (CoA) is formed from propionyl-CoA by the enzyme propionyl-CoA carboxylase mediated by biotin (vitamin B7). One approach is to use labeled sodium propionate as a feed to incorporate the label, either via propionyl-CoA or methylmalonyl-CoA into the propionate backbone of the molecule. Before [14C] radiolabelling starts, initial studies are carried out using sodium [1-13C]propionate. The labelled substrate is used in a feeding strategy to incorporate the carbon-13 label into the polyketides molecule mediated by a suitable microorganism (streptomyces sp).

Control of physical form

Control of physical form is a major issue in the development of any new chemical entity. Almac has expertise in the control of polymorphic form by the development of optimum crystallisation conditions which spans into isotope chemistry. A typical project involved confirmation that the correct polymorph was achieved from the [14C] synthesis as was achieved from the cold synthesis using XRPD analysis. Over-layed XRPD traces of both the hot and cold API showed identical structure and therefore they have the same polymorph. The [14C] API was then formulated into drug product and shipped to the customer. In addition to XRPD analysis, particle size can be controlled and confirmed by the use of milling and microscopy, respectively.

In conclusion

Almac's isotope chemistry clearly benefits from the active integration of technology platforms from biocatalysis, physical sciences and peptide synthesis to prepare diverse isotopically labeled products as highlighted in this article.

The future prospects of drug development and the complexity of products is at the forefront of science and is captivating new limits of what is possible at the interface of scientific fields. This in turn is leading to more isotopic labelling challenges. To overcome this, isotope chemistry must now work in tandem with different technology fields if they are to be successful in aiding drug development with pharmaceutical companies. Almac is continuing to diversify its offering and continues to lead in the area of complex isotopic labeling services. The future is certainly exciting.

This article is by Dr Tom Moody, Dr Sean L. Kitson, Dr Derek J. Quinn, Dr David Speed, Dr Iain Miskelly and Dr William Watters of Almac.

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