Point of VIEW

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Ester Bonds in Prodrugs

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ABSTRACT A recent study challenges the oft-held notion that ester bonds in prodrug molecules are cleaved rapidly and completely inside cells by endogenous, nonspecific esterases. Structure–activity relationship studies on acylated sugars reveal that regioisomeric compounds display disparate biological activity, suggesting that ester bonds can persist in a cellular context.

Cell biology is a veritable concert of enzymatic activity functioning within subcellular domains. Chemical biologists often rely on this collection of cellular enzymes to facilitate the delivery, and occasionally the installation, of small molecules within cells. An example is the area of "metabolic glycoengineering", where endogenous sugar-processing pathways are exploited to incorporate non-natural sugars into glycans for targeting, labeling, and other useful biological experiments (*1*). The paper by Yarema and co-workers on page 230 of this issue describes structure–activity relationship (SAR) studies on acylated carbohydrates for use in metabolic glycoengineering (*2*). The data indicate that the position of acyl groups affects strongly the biological activity of the carbohydrate.

Fashioning bioavailable molecules is central to experiments on the brink between chemistry and biology. This is particularly true for metabolic glycoengineering, as the numerous polar functionalities in carbohydrate derivatives can render such molecules cell-impermeant. The success of this metabolic engineering technique has led to investigations of cellular delivery strategies for simple carbohydrates. A common solution to the permeability problem involves the acylation of the sugar hydroxyl moieties with short chain fatty acid (SCFA) groups. This chemical modification can enhance uptake by up to 3 orders of magnitude (*3*). The nonpolar acylated sugars presumably cross lipid bilayers and are assumed to be unmasked *via* endogenous esterase activity prior to utilization by native metabolic pathways.

Yarema and co-workers (*2*) point out that this esterase-activated delivery strategy can complicate biological studies, an important reminder to chemical biologists building and using masked molecules. For example, hydrolysis of an SCFA–sugar prodrug releases unfettered SCFA molecules such as butyric acid. These compounds are known to exert biological activities on their own, such as inhibition of histone deacetylase activity (*4*). To deconvolute and better control the biological activities of these compounds, the authors omitted a single butyrate (Bu) from perbutanoylated *N*-acetyl-D-mannosamine (ManNAc), compound **1**, at either C-1 (**2**) or C-6 (**3**) as shown in Figure 1. Assuming rapid action of cellular esterases, one might expect only superficial differences between the biological activities of the two isomers. Surprisingly, the activity of the two sugar derivatives diverged. Compounds with a free hydroxyl group at position C-1 (**2**) showed moderate cytotoxicity, whereas a free hydroxyl group at C-6 (**3**) afforded relatively low toxicity and efficient incorporation into cellular glycans. This pattern of biological activity held for analogous regioisomers of other acylated sugars. Overall, this report validates the utility of organic chemistry and careful SAR studies to discover new structure–activity motifs in small molecules.

From the point of view of an organic chemist working on the interface of chemistry and biology, this study also raises important questions about the methods employed to deliver small molecules to cells. Many biologically active small molecules are often too hydrophilic to cross cell mem-

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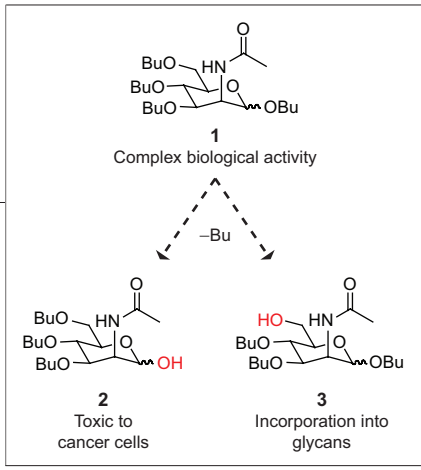

Figure 1. ManNAc derivatives 1–3 from Yarema and co-workers (*2*). Peracylated sugar 1 incorporates into cellular glycans at relatively low concentrations but exhibits cytotoxicity at higher levels. Omission of one ester moiety at C-1 (compound 2) yields a molecule with enhanced cytotoxicity. A sugar derivative presenting a free hydroxyl at C-6 (compound 3) displays negligible cytotoxicity and is incorporated into cellular glycans with high efficiency.

branes. As mentioned above, one common solution to this problem involves the chemical masking of polar moieties as esters and invoking the collection of endogenous, nonspecific esterases to nake the molecule inside the cell. Yarema and colleagues note that enzyme-catalyzed hydrolysis of the ester groups in SCFA–sugars is often assumed to be faster than ensuing biological activity. Their findings, however, stand in opposition to this paradigm. The difference in activity between sugar derivatives **2** and **3** suggests that these synthetic ester bonds are not hydrolyzed immediately to yield free ManNAc. Instead, ester moieties must endure inside cells and influence biological activity (*2*).

The esterase-mediated strategy to unmask compounds is pervasive in medicinal chemistry and chemical biology. In addition to masking alkyl hydroxyl groups found in sugar derivatives, ester moieties are often used to shroud other polar functionalities such as aryl hydroxyl groups, carboxyl moieties, and even amines (*5*). In many cases, this strategy yields prodrugs with improved properties both *in cellulo* (*6*) and *in vivo* (*7*). In particular, the use of ester-containing prodrugs *in vivo* can bring about improved bioavailability; the removal of ester functionalities is accomplished through enzyme catalysis or spontaneous hydrolysis. An example of a successful pharmaceutical masked with an ester group is enalapril (**4**), the structure of which is shown in Figure 2 (*8*).

Another manifestation of esterase-mediated delivery involves fluorescent dyes. Fluorogenic molecules (*i.e.*, "profluorophores") that are shrouded by ester moieties comprise an important use of esterase-mediated unmasking of small molecules. Decades ago, Rotman and Papermaster (*9*) showed that the colorless fluorescein diacetate (**5**; Figure 2) could cross cell membranes and be converted to the fluorescent dye fluorescein. This seminal study is cited as a substantiation of the presence of promiscuous esterases in different cell types (*10*). Today, the use of fluorescein diacetate and its derivatives as fluorogenic esterase substrates is the basis for a number of cellular assays for viability and long-term tracking (*11*). More recently, other esterase-active profluorophores have been introduced based on aryl acetate esters possessing higher chemical stability (*12*).

In addition to fluorogenic esterase substrates, esters are also used to mask carboxyl groups in fluorescent ion indicators. The vastly important calcium ion indicators developed by Tsien and co-workers (*13, 14*) proved difficult to deliver to the interior of cells. Inspired by β-lactam prodrugs (*15*), Tsien demonstrated that the carboxyl functionalities in these indicators could be cloaked as acetoxymethyl (AM) esters (*10*). The hydrolysis of the acetate ester followed by spontaneous hydrolysis of the nascent hemiacetal gives the free carboxyl group. AM groups can also be used to mask phenolic moieties to prepare prodrugs (*16*) and profluorophores (*17*). An example of this strategy in a compound available commercially is the calcium indicator Fluo-3 AM (**6**) in which the four carboxyl groups on the calcium-chelating 1,2-bis(*o*-aminophenoxy)

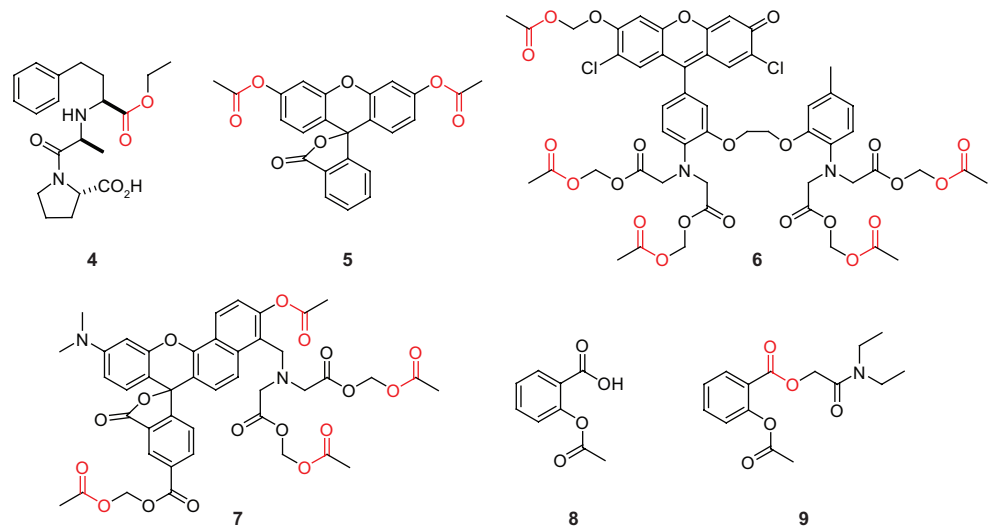

Figure 2. Ester-containing prodrugs and profluorophores: enalapril (4), fluorescein diacetate (5), Fluo-3 AM (6), C-SNARF-1–calcein AM (7), aspirin (8), and aspirin *N,N*-diethylglycolamide ester (9). Critical ester bonds are indicated in red.

ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) moiety and the phenol on the fluorophoric portion of the indicator are masked using AM groups (*14*).

The synopsis above demonstrates the utility of esterase-mediated hydrolysis to deliver pharmacologically active molecules and fluorescent compounds into cells. Interestingly, the reliance of esterases in such prodrug strategies is juxtaposed with rather incomplete information about the cohort of esterases in biological systems. Despite the considerable body of research performed on esterases (*5, 18–20*), this class of enzymes remains difficult to categorize because of the promiscuity and polymorphism of these proteins (*5*). Different classification schemes exist that group esterases according to interaction with organophosphates or sequence identity (*20*). A classic discrimination of esterases involves the term "nonspecific", which traditionally defines an esterase that cleaves synthetic substrates such as simple naphthyl esters (*18, 19*).

The poorly defined substrate specificity of esterases is exacerbated by the wide distribution of esterases *in vivo*. Esterases are found in a number of tissues, particularly the liver, kidney, and plasma (*5, 19, 20*). These enzymes are thought to play an important role in the detoxification of xenobiotics, perhaps explaining the promiscuity and distribution of these enzymes (*20*). Although ester formation can improve global bioavailability of pharmaceuticals, this ubiquity of esterases frustrates the development of tissue-selective prodrugs that rely on esterase-mediated cleavage (*21*). Beyond the tissue distribution of this enzyme family, the subcellular localization of these enzymes is also vital to understanding the differential activity of esterase-masked compounds. Again, rigorous determination of cellular esterases is complicated by the sheer number of enzymes exhibiting this activity. A recent survey of the location of numerous enzymes within mammalian cells (*22*) indicates the presence of carboxylesterases in the cytosol and endoplasmic reticulum. This sequence-based study correlates with activity-based studies that also show heterogeneous distribution of esterase activity (*20*).

The varying distribution and selectivity of different esterases inside cells could explain the differential activity of sugar derivatives **2** and **3** reported by Yarema and co-workers. Other studies also raise questions about the utility of endogenous esterases to unmask ester-containing molecules. Webb and colleagues demonstrated that fluorescent indicators for pH delivered *via* neutral AM esters such as seminaphthorhodafluor (SNARF) derivative **7** exhibit vesicular localization (*23*). Another classic example of ester bonds persisting in a biological context is acetylsalicylic acid (aspirin; **8**). Aspirin can be hydrolyzed by endogenous esterases to salicylic acid, and both compounds exhibit interesting biological activities (*24*). The well-known irreversible inhibition of cyclooxygenase (COX) by aspirin, however, requires the acetate ester to acetylate a particular serine residue (*25*). Thus, a "true" aspirin prodrug requires a molecule with a benzoic acid ester moiety that is hydrolyzed more quickly than the requisite phenol acetate, as seen with glycolamide ester **9** (*26*).

For research on the boundary of chemistry and biology, the issue of delivery of small molecules to the biological world is paramount. The Yarema study raises important issues for the field of chemical biology, reminding us that masking small molecules with enzyme-labile groups is not always a transparent endeavor. This result is corroborated by other reports that suggest differential hydrolysis in subcellular compartments and the persistence of ester bonds in biological environments. Although esterase-mediated delivery strategies will (and should) continue to be utilized, we must bear in mind that the collection of esterases in biology is heterogeneous with ill-defined specificity. Thus, subtle changes in ester-masked compounds can yield large differences in biological activity. Organic synthesis and careful SAR studies can help evaluate prodrug strategies and, as in the case of Yarema and co-workers, lead to the discovery of small molecules with nuanced biological activity.

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