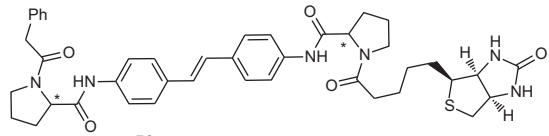


**50a**: (S,S at C*) G-1b $EC_{50}$ = 33 nM
**50b**: (R,R at C*) G-1b $EC_{50}$ > 10 μM

**Figure 1.14** Biotin-tagged tool compounds.

proteins and **50a** failed to pull down the NS5A protein of BVDV under similar conditions.

The availability of potent NS5A-targeting tool compounds has catalyzed additional efforts that are directed at shedding light on the ramifications of inhibition of the NS5A protein which, in turn, provides additional insights into the mode of action of the inhibitors. NS5A is a phosphoprotein that exists in both basally phosphorylated (p56) and hyper-phosphorylated (p58) forms. It was discovered that NS5A inhibitors such as daclatasvir (**1**) dose-dependently inhibit the formation of the p58 form, without affecting basal phosphorylation, in a manner that correlates with their RNA replication-inhibitory activities.[19,90] Moreover, inhibition of hyperphosphorylation did not depend on the presence of Domains II and III of NS5A. Although the exact significance of phosphorylation of NS5A is still unknown, it is hypothesized that phosphorylation is a regulatory mechanism that toggles the NS5A protein between functional states in the viral replication cycle. Interestingly, it was observed that protease inhibitors could similarly inhibit the formation of p58 in a dose-dependent manner, clearly indicating that the modulation of the phosphorylation state of NS5A is not unique to NS5A inhibitors, albeit this observation may signify the spatial and functional associations of the NS3 and NS5A proteins within the HCV replication complex or polyprotein.[19] Corroborating evidence for a possible interaction of NS5A inhibitors at the polyprotein-processing stage came from a recent study that demonstrated that treatment with **1** results in the accumulation of the NS4B–NS5A polypeptide *in vitro*, an effect that was sensitive to the presence of resistance-conferring mutations.[90]

A combination of morphological and biochemical studies have demonstrated that **1** alters the subcellular distribution of NS5A from that of localized foci to diffuse cytoplasmic patterns.[88] This inhibitor-induced effect on the subcellular disposition was specific to the NS5A protein and, in line with expectations, was minimized in a replicon harboring the Y93H-resistant mutant up to certain concentration ranges. A different set of studies revealed that NS5A inhibitors deregulate the normal distribution of NS5A by relocating the protein from the endoplasmic reticulum to lipid droplets, an effect that is minimized in the context of the Y93H-resistant mutant and which is also specific to the NS5A replication complex inhibitor class.[89] Finally, a cellular imaging study that utilized a click chemistry approach involving an azide-containing NS5A