Chemical Dissection of Sterol and Bile Acid Signaling via Clickable Photoaffinity Probes

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Abstract

Sterols and bile acids, traditionally viewed as structural components and digestive surfactants, have emerged as critical signaling molecules involved in diverse physiological processes, including metabolism, immunity, cancer, and host–microbiota interactions. Despite their importance, the full spectrum of proteins that interact with these metabolites remains incompletely defined. Clickable photoaffinity probes have revolutionized our ability to systematically profile sterol and bile acid-binding proteins in living systems. These bifunctional probes, incorporating a photo-crosslinker and a bioorthogonal handle, enable covalent capture and enrichment of probe-interacting proteins for proteomic identification and binding site mapping. Here, we review the design principles and applications of sterol and bile acid probes in target identification and mechanistic studies. We highlight key discoveries that illuminate how cholesterol and bile acid derivatives engage diverse protein targets, including nuclear receptors, ion channels, transporters, and virulence regulators, to influence cellular and microbial physiology. Together, these advances underscore the power of chemical biology tools to decode metabolite signaling networks and identify therapeutic targets at the interface of metabolism, immunity, cancer, and microbial ecology.

Introduction

Sterols are essential structural lipids found in the membranes of most eukaryotic cells (Rohman and Dijkstra 2021). Characterized by a rigid steroid nucleus composed of four fused rings (three cyclohexane and one cyclopentane), sterols represent a unique molecular scaffold in biology. The prototypical sterol in animal tissues is cholesterol,

an amphipathic molecule with a polar hydroxyl group at C3 and a hydrophobic hydrocarbon tail (**Figure 1**). Cholesterol-derived bile acids are constituted by primary bile acids and secondary bile acids based on their biosynthetic origin. Primary bile acids are synthesized in the liver from cholesterol and subsequently stored in the gallbladder (Hofmann 1999). Upon food intake, primary bile acids are secreted into the small intestine, where they facilitate the emulsification and absorption of dietary oil and fats. Microbial enzymes in the gut further convert primary bile acids into a structurally diverse pool of secondary bile acids, which can have distinct physiochemical properties (Dawson and Karpen 2015; Di Ciaula *et al.* 2017; Hofmann and Hagey 2014a; Ridlon *et al.* 2006; Sjövall 2004). For example, bile salt hydrolases (Yao *et al.* 2018) convert conjugated bile acids into free bile acids, which can then be reconjugated with amino acids (Fu *et al.* 2023; Mohanty *et al.* 2024b; Quinn *et al.* 2020; Rimal *et al.* 2024), polyamines (Mohanty *et al.* 2024b) or other chemical groups (Won *et al.* 2025). In addition, the 3-hydroxyl group can undergo further modifications, such as succinylation or acylation, leading to the formation of 3-succinylated (Nie *et al.* 2024) or 3-acylated cholic acids (Liu *et al.* 2024) (Figure 1). A comprehensive bile acid profile is summarized in other elegant reviews.(Hofmann and Hagey 2014b; Mohanty *et al.* 2024a)

Beyond serving as structural or digestive molecules, sterols and bile acids play important roles in signaling and physiological regulation. Cholesterol is not only an essential component of membranes but also modulate membrane fluidity (Sviridov *et al.* 2020). Meanwhile, cholesterol also serves as a biosynthetic precursor for bioactive steroids, such as steroid hormones (Hu *et al.* 2004), vitamin D (Christakos *et al.* 2016), and bile acids (Hofmann 1999). Cholesterol also binds and affects different receptors, such as ion channels (GABA receptor) (Belelli and Lambert 2005; Lambert *et al.* 2009; Mitchell *et al.* 2008), nuclear receptor (Estrogen-related receptor alpha) (Casaburi *et al.* 2018; Wei *et al.* 2016). Oxidized derivatives of cholesterol, such as 25-hydroxycholesterol (25-HC), act as potent signaling molecules that regulate lipid metabolism and immune responses (Cao *et al.* 2020; Cyster *et al.* 2014). For instance, 25-HC suppresses cholesterol synthesis by inhibiting sterol regulatory element-binding proteins (SREBPs) (Adams *et al.* 2004; Trindade *et al.* 2021). For decades, bile acids were primarily regarded as physiological surfactants facilitating the emulsification and absorption of dietary lipids. However, following the seminal discovery of bile acid-activated nuclear receptor farnesoid X receptor (FXR) (Makishima *et al.* 1999; Parks

et al. 1999; Wang *et al.* 1999) and membrane receptor Takeda G-protein receptor 5 (TGR5) (Kawamata *et al.* 2003; Maruyama *et al.* 2002), contemporary research has established their critical role as signaling molecules that modulate both nuclear receptor-mediated transcriptional regulation and membrane receptor-dependent cell signaling pathways. Subsequent research has further expanded the bile acid receptor repertoire to include the vitamin D receptor (VDR) (Adachi *et al.* 2005; Makishima *et al.* 2002; Nehring *et al.* 2007), steroid and Xenobiotic receptor (SXR)/pregnane X receptor (PXR) (Staudinger *et al.* 2001; Xie *et al.* 2001), S1PR2 (Liu *et al.* 2014; Nagahashi *et al.* 2015; Studer *et al.* 2012), LXR (De Marino *et al.* 2017; Janowski *et al.* 1996), Muscarinic M2/M3 (Ibrahim *et al.* 2018; Sheikh Abdul Kadir *et al.* 2010; Urso *et al.* 2020) and CAR (Chen *et al.* 2021; Kovács *et al.* 2019), thereby influencing a wide range of physiological processes such as glucose and lipid metabolism (Chaudhari *et al.* 2021; Sun *et al.* 2018), inflammation (Jia *et al.* 2018; Lee *et al.* 2024), and tumorigenesis (Cai *et al.* 2022; Varanasi *et al.* 2025). Recently, structurally diverse bile acids are also connected to other protein target such as MRGPRX4 (Meixiong *et al.* 2019; Yang *et al.* 2024; Yu *et al.* 2019), ROR γ t (Hang *et al.* 2019; Paik *et al.* 2022; Song *et al.* 2020), NR4A1 (Li *et al.* 2021), TULP3 (Qu *et al.* 2024b), PMCA (Cong *et al.* 2024), AR (Jin *et al.* 2025), STING (Yang *et al.* 2025) and MRGPRE (Lin *et al.* 2025). Among these, FXR has been identified as a key drug target in clinical therapy. Obeticholic acid (OCA), a bile acid derivative and an FXR agonist, has been used to treat Primary Biliary Cholangitis (Schaap *et al.* 2014). The identification of the bile acid receptor MRGPRX4 has enabled the development of an OCA derivative that effectively treats liver diseases while avoiding the pruritus side effect commonly associated with bile acid therapies (Yang *et al.* 2024). Also, STING has been recognized as a critical component in the innate immune response. Recent studies have highlighted its potential as a therapeutic target for various inflammatory diseases and cancer (Lanng *et al.* 2024). Given the unique structural diversity and broad biological functions of sterols and bile acids, it is highly probable that their regulatory effects are mediated through interactions with numerous proteins. Therefore, comprehensive profiling of sterol- and bile acid-binding proteins is not only essential for understanding their physiological roles but could also significantly advance bile acid-related drug discovery.

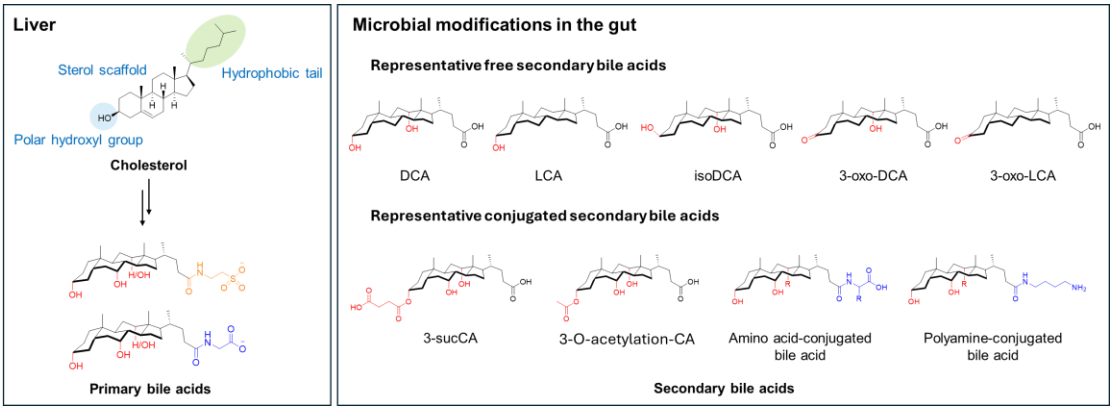

Figure 1. Representative chemical structures of cholesterol and bile acids.

Clickable Photoaffinity Probe

Exploring the potential non-covalent interactions between small molecules and target proteins has gained more and more attention recently. Probe-free chemical proteomics has seen significant development in recent years (Wang *et al.* 2025). These approaches mainly rely on detecting changes in the biophysical properties of proteins—such as thermal stability (Huang *et al.* 2019), resistance to chemical denaturants (Van Vranken *et al.* 2021; Zhang *et al.* 2020a), and susceptibility to proteolysis—before and after ligand binding (Piazza *et al.* 2018; Tian *et al.* 2023), etc. By analyzing these changes at the proteome level upon addition of small-molecule metabolites, intracellular binding proteins can be identified without the need for specially designed chemical probes. However, these methods also face several limitations. Firstly, small molecule–protein interactions can trigger downstream signaling events that alter the physicochemical properties of non-binding proteins, potentially leading to false positives. Secondly, interactions involving covalent binding, weak affinity, or substoichiometric ratios may not induce detectable changes in protein properties. Moreover, because these methods lack an enrichment step, low-abundance proteins may be overlooked, resulting in false negatives. Thirdly, these techniques generally require large sample quantities and demand high reproducibility across replicates. Clickable photoaffinity probes have emerged as powerful chemical tools for the unbiased mapping biomolecule interactions, such as small molecule-protein interaction (Gao *et al.* 2024; Ge *et al.* 2022; Grammel and Hang 2013; Homan *et al.* 2024; Parker and Pratt 2020; Qin *et al.* 2020; Tsukidate *et al.* 2020; West and Woo 2023; Yu and Baskin 2022), protein-protein interaction (Lin *et al.* 2021; Murale *et al.* 2016) etc. Specifically, small molecule probes operate within

the framework of chemical proteomics, enabling the global profiling of noncovalent small molecule-protein interaction in complex biological systems (Malarney and Chang 2023; Weigert Muñoz *et al.* 2024; Zhang *et al.* 2020b; Zhao *et al.* 2022).

Photoaffinity probes incorporate latent reactive moieties that, upon irradiation at specific wavelengths, generate highly reactive intermediates capable of forming covalent bonds with proximal biomolecules. When integrated into a bioactive small molecule, PAL enables the covalent capture of transient molecular interactions within native cellular environments, preserving physiological protein conformations and endogenous binding networks. Despite its advantages, PAL still faces several limitations that hinder its broad applicability. First, the rational design of photoactive probes requires careful consideration of the ligand's structure-activity relationship (SAR), as the incorporation of photo-reactive and bio-orthogonal functional groups may perturb the molecule's binding affinity and target specificity. Second, the chemical synthesis of such probes is technically challenging and often lacks generalizability across diverse metabolites of interest. In this review, we focus on the chemical dissection of sterol and bile acid signaling using clickable photoaffinity probes. These powerful tools enable the systematic investigation of sterol- and bile acid–interacting proteins in both mammalian cells and bacteria. The general workflow involves incubating cells or bacteria with the probe, UV-induced covalent crosslinking of transient interactions, and enrichment of labeled proteins via bioorthogonal chemistry, followed by imaging, mass spectrometry-based protein identification, and binding site mapping (**Figure 2A**).

Photoaffinity probe design

A typical photoaffinity probe comprises three essential components: 1) Recognition Element: This component is derived from a bioactive sterol or bile acid and is responsible for recapitulating the biological activity and target engagement of the native molecules.

2) Photo-crosslinker: Several photo-crosslinker moieties varying in size and photo-reactivity has been utilized in probe design (**Figure 2B**), such as aryl azides (Borden *et al.* 2000; Fleet *et al.* 1969), benzophenones (Dormán *et al.* 2016; Galardy *et al.* 1974), diazirines (Brunner 1993; Das 2011; Smith and Knowles 1973; West *et al.* 2021) and aryl tetrazoles (Herner *et al.* 2016; Li *et al.* 2016). Diazirine is one of the most commonly used moieties in photoaffinity labelling because of its minimized size,

relatively hydrophobic, high photoreactivity and considerably longer wavelengths that reduce protein damage. Upon UV irradiation (typically at 365 nm), diazirine generates a diazo intermediate that subsequently eliminates nitrogen to form a highly reactive carbene intermediate, which can covalently crosslink with proximal amino acid residues within the binding site (Brunner 1993; West *et al.* 2021).

3) Clickable Handle: Typically an alkyne or azide, this bioorthogonal functional group enables post-crosslinking conjugation to reporter tags (e.g., biotin or fluorophores, depending on the experimental needs) via copper-catalyzed azide-alkyne cycloaddition (CuAAC) (Kolb *et al.* 2001), facilitating downstream enrichment and visualization. This modular design not only ensures target engagement but also enables proteome-wide mapping of interactions and site-specific identification at the amino acid level through tandem mass spectrometry (Parker and Pratt 2020; Scinto *et al.* 2021; Speers and Cravatt 2004; Yang *et al.* 2013).

Probe design requires careful balance between chemical functionality and biological activity. The structure and position of photo-crosslinker and clickable handle on recognizition element scaffold can directly impact probe biological activity (Conway *et al.* 2021). The most straightforward approach is to integrate both the photo-crosslinker and the clickable handle into a single linker, which is appended onto the sterol or bile acid scaffold. However, this strategy can sometimes disrupt native bioactivity due to steric or electronic interference (Conway *et al.* 2021). As an alternative, the two functional groups, diazirine and alkyne/azide, can be introduced separately. For instance, diazirines can be introduced via functional group transformation from ketones on the sterol ring, while alkyne handles can be installed at sites distal to the ligand-binding pharmacophore to preserve biological function (Hulce *et al.* 2013).

In addition to protein target discovery, clickable photoaffinity probes are invaluable for identifying the exact binding site of a small molecule on its protein target (Gertsik *et al.* 2017; Korovesis *et al.* 2023; Parker *et al.* 2019; Verhelst *et al.* 2007; Wozniak *et al.* 2024). Enriched probe-labelled peptides are cleaved, followed by proteolytic digestion and mass spectrometric analysis, enables pinpointing of crosslinked peptides, offering mechanistic insights into small molecule recognition and functional modulation (**Figure 2C**).

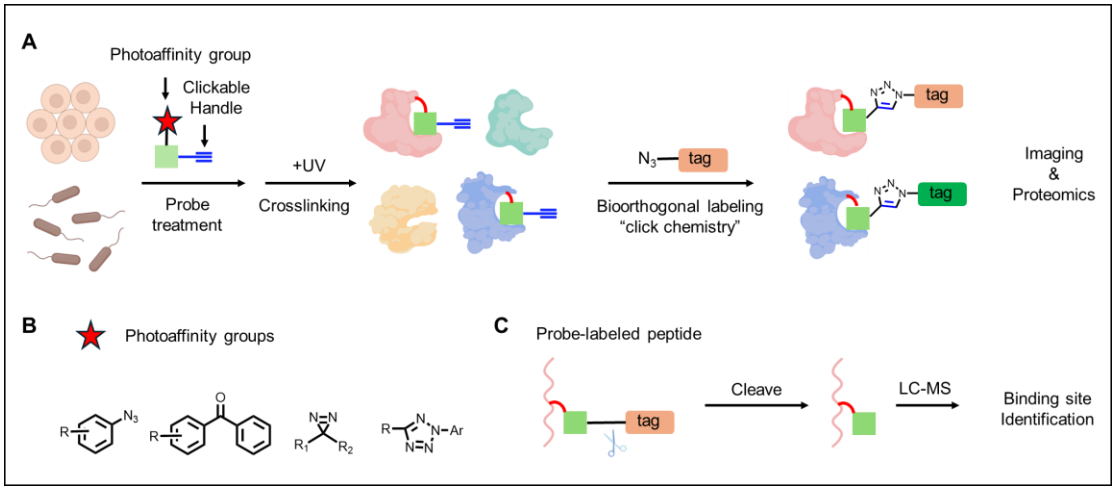

Figure 2. Design of a typical clickable photoaffinity probe and schematic workflow for identifying sterol and bile acid interacting proteins. (A) Proteomics workflow illustrating enrichment, identification, and binding site mapping of sterol and bile acid targets using mass spectrometry. (B) Samples of photo-crosslinker moieties used in photoaffinity probe, including aryl azides, benzophenones, diazirines and aryl tetrazoles. (C) workflow for identification of the exact binding site for a small molecule on its protein target.

Applications in Target Identification and Mechanistic Studies

Given the unique molecular scaffolds and critical biological functions of sterols and bile acids, systematic proteome-wide identification of their interacting proteins is essential. To characterize these interactions comprehensively, multiple studies have employed chemical proteomics strategies, designing bifunctional clickable photoaffinity probes for target identification and mechanistic investigations. These approaches have enabled the discovery of numerous sterol/bile acid-binding proteins, including receptors, ion channels, and enzymes. While some interactions were previously known, many represent novel findings, significantly expanding our understanding of sterol and bile acid signaling in cellular regulation.

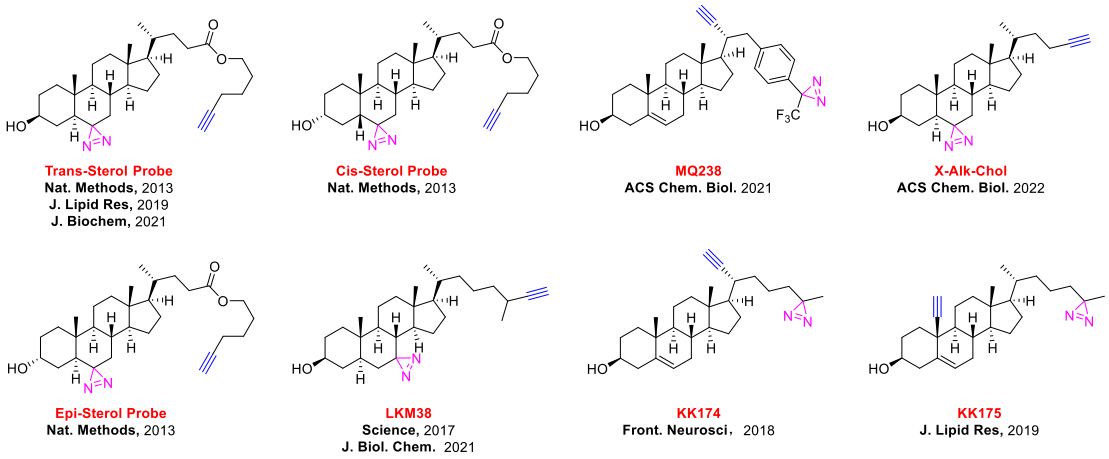

Figure 3. Chemical structure of representative clickable photoaffinity probes designed for cholesterols.

Sterol probes

The Cravatt lab (Hulce *et al.* 2013) developed a chemoproteomic approach using photoreactive sterol probes and quantitative mass spectrometry to systematically map cholesterol-protein interactomes in living cells (**Figure 3**). In this case, a diazirine moiety was strategically placed at the C-6 position of cholesterol, a site demonstrated to have minimal impact on the biophysical properties and membrane behavior of natural molecule. An alkyne group was introduced into the alkyl side chain of cholesterol via an ester linkage to simplify the synthetic process. Although this ester linkage was expected to change the polarity of the alkyl side chain, the authors prioritized this modification to facilitate recognition of most cholesterol-binding proteins rather than introducing additional perturbations to the steroid core. Then the analysis identified >250 cholesterol-binding partners, including canonical sterol regulators (receptors, channels) and novel interactors in metabolic control (sugar/glycerolipid/cholesterol enzymes), cellular trafficking (vesicular transport proteins) and protein homeostasis (glycosylation/degradation machinery). These findings reveal previously unrecognized sterol-sensing nodes that may coordinate metabolite coupling (sterol/lipid/sugar cross-regulation) and spatial control of protein localization/modification.

In 2017, the Zoncu lab (Castellano *et al.* 2017) elucidates cholesterol as a critical nutrient signal for mTORC1 activation at lysosomes. To investigate the interaction between lysosomal transmembrane protein (SLC38A9) and cholesterol, they

synthesized a photoactivatable cholesterol analog (LKM38) for biochemical characterization (**Figure 3)**. They identified lysosomal cholesterol activates mTORC1 via SLC38A9- Niemann-Pick C1 (NPC1) signaling complex.

In the same year, the Evers lab (Budelier *et al.* 2017) identified specific cholesterol-binding sites in mouse VDAC1 (mVDAC1) using photoactivatable cholesterol analogs (**Figure 3**: LKM38 and KK174) coupled with top-down and bottom-up mass spectrometry (MS) enhanced by a clickable isotope-labeled FLI-tag. For synthetic considerations and to investigate differences in the contacting amino acid residues at distinct sites on the molecule for mapping the cholesterol binding pocket, the authors introduced the diazirine moiety at either the steroid core (LKM38) or the side chain (KK174) of cholesterol. These findings suggest that cholesterol may regulate VDAC1 function through a specific interaction at Glu73, a critical residue for channel activity. The study provides structural insights into cholesterol-dependent modulation of mitochondrial membrane proteins. In 2019, the Schaffer lab (Feltes *et al.* 2019) systematically characterized a panel of diazirine-alkyne cholesterol probes (LKM38, KK174, KK175, and trans-sterol) for investigating cholesterol biology (**Figure 3**). Through comprehensive evaluation in cholesterol-auxotrophic cells, the authors identify LKM38 as the most faithful cholesterol mimic, capable of supporting cell growth and properly modulating cholesterol homeostatic pathways.

Using a photoreactive clickable cholesterol analog (**Figure 3**: Trans-Sterol Probe) in MA-10 Leydig cell mitochondria, the Papadopoulos lab (Georges *et al.* 2021) provided direct biochemical evidence that the mitochondrial translocator protein (TSPO) binds cholesterol through physiologically relevant interactions. These findings establish TSPO as a bona fide cholesterol-binding protein and validate PhotoClick cholesterol as a tool for studying sterol-protein interactions. The work resolves longstanding questions about TSPO-cholesterol binding while providing a methodological framework for investigating membrane protein-lipid interactions.

The structure of photo-crosslinker and clickable handle on recognizition element scaffold can directly impact probe biological activity. Aryl trifluoromethyl diazirines predominantly react via carbene intermediates, in contrast to the diazo intermediates typically observed with aliphatic diazirines. This mechanistic divergence results in distinct labeling efficiencies between the two photoprobe classes. The Covey lab (Krishnan *et al.* 2021) systematically compares cholesterol-based probes containing either trifluoromethylphenyl diazirine (TPD) or aliphatic diazirine moieties (**Figure 3**

& Figure 4) for mapping cholesterol-binding sites in the Gloeobacter ligand-gated ion channel (GLIC). To preserve biological activity when introducing the large group TPD moiety into cholesterol, they incorporated TPD group at the iso-octyl position of the side chain rather than the steroid core of cholesterol, therefore synthesizing three classes of TPD-containing probes. The authors demonstrate that all the probes bound to the same cholesterol binding site on GLIC but with different labeling efficiencies and residues identified.

The Hang lab (Das *et al.* 2022) used cholesterol photoaffinity probes (**Figure 3**: X-Alk-Chol) elucidating the mechanistic interplay between S-palmitoylation and cholesterol binding in regulating the antiviral function of interferon-induced transmembrane proteins (IFITMs). As for the design of probe, the authors drew lessons from the existing cholesterol probe designs reported in the literature and found that x-alk-chol showed similar molecular topology as cholesterol through maestro modeling. These findings establish a palmitoylation-cholesterol axis as a tunable mechanism controlling IFITM's membrane topology and antiviral spectrum, offering new insights for developing broad-spectrum antiviral strategies targeting host lipid pathways.

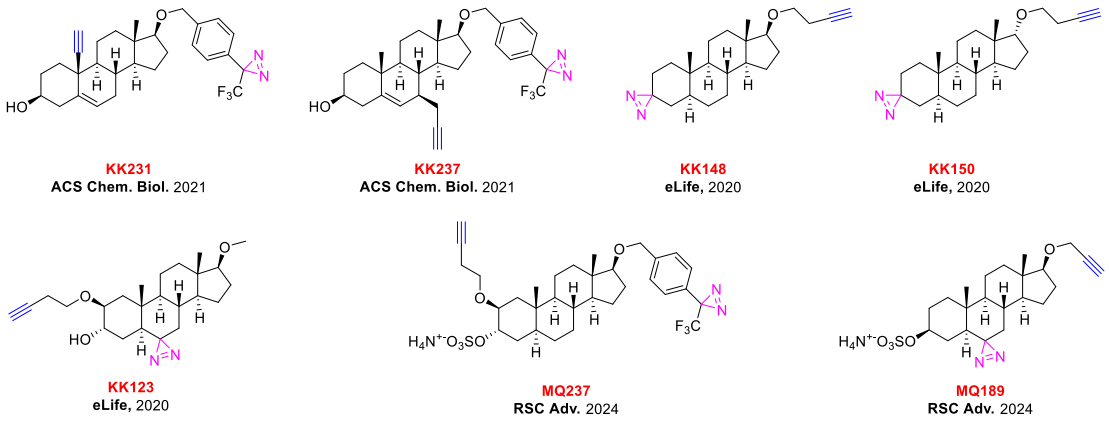

Figure 4. Chemical structure of representative clickable photoaffinity probes designed for steroids.

Neurosteroids (NS) are endogenous neuromodulators that critically regulate brain development, neural function, and mood homeostasis. Clinically, synthetic NS analogues have been utilized as anesthetic and antidepressant agents, while emerging evidence supports their therapeutic potential in epilepsy management, neuroprotection, and cognitive enhancement. In 2020, the Evers lab (Sugasawa *et al.* 2020) used allopregnanolone and synthetic diazirine-containing neurosteroid analogs (**Figure 4**:

KK148/KK150) to elucidate the structural basis of neurosteroid modulation of α1β3 $GABA_A$A receptors through three distinct binding sites. Through comparative analysis of binding versus functional outcomes, this work establishes that the integrated activity across these three sites determines whether neurosteroids act as potentiators, inhibitors, or antagonists. Their findings provide a structural framework for developing targeted $GABA_A$A receptor modulators and reveal KK148/150 as potential scaffolds for neurosteroid antagonists.

Lately, the Covey lab (Qian *et al.* 2024) reports the synthesis and electrophysiological evaluation for a panel of sulfated steroid (pregneolone sulfate and dehydroepiandrosterone sulfate) (**Figure 4**) probes for photolabeling of NMDARs and $GABA_A$Rs. These are the first sulfated probes reported for use in photolabeling experiments to identify binding sites for DHEAS and/or PS on either of these receptors. Some of these probes may also find use in identifying sulfated steroid binding sites on other type of receptors.

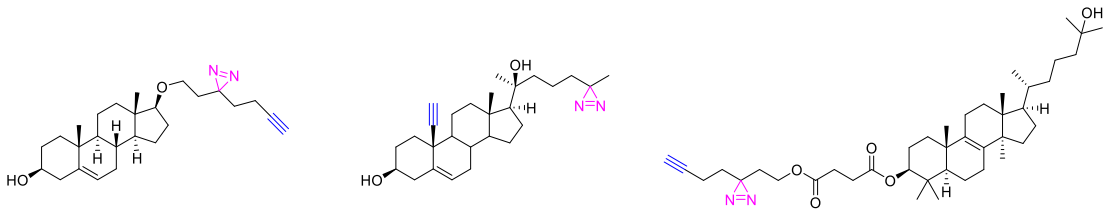

Figure 5. Chemical structure of representative clickable photoaffinity probes designed for oxidized sterols.

Oxidized sterol probes

Oxidized derivatives of cholesterol act as potent signaling molecules that regulate lipid metabolism and immune responses. To investigate oxysterol signaling, the Mennerick lab and Coey lab (Chen *et al.* 2018) designed a bifunctional oxysterol probe (**Figure 5**: MQ182). The probe retains native oxysterol activity at NMDA receptors and exhibits competitive binding with endogenous oxysterols. Live-cell imaging revealed energy-independent cytoplasmic accumulation in neurons. This work establishes a versatile tool for spatial mapping and molecular identification of oxysterol-protein interactions, enabling future mechanistic studies of oxysterol signaling pathways. The modular design principle may be extended to other sterol derivatives.

In 2021, the Ondrus lab (Cheng *et al.* 2021) developed a chemoproteomic strategy

to systematically map protein targets of 20(S)-hydroxycholesterol (20(S)-OHC) in living cell membranes. Using a precision-designed chemical probe (**Figure 5**) that preserved the α - and β -face topologies of the tetracyclic ring system, the accessible C3 hydroxyl group and the length and hydrophobicity of the C17 side chain, they identify a proteome-wide interaction network linking 20(S)-OHC to immune regulation and cancer pathways. Through competitive profiling, they establish 20(S)-OHC as a highly selective ligand for TMEM97/σ2 receptor, resolving its binding mode with chemo-, regio-, and stereospecificity. By integrating quantitative target engagement analysis with structural mapping, this work both reveals broader biological roles of oxysterols and identifies TMEM97 as a druggable target for 20(S)-OHC signaling. In 2022, the Qi lab (Jiang *et al.* 2022) identified 25-hydroxylanosterol (25-HL) as a potent SREBP inhibitor with therapeutic potential for metabolic disorders. They synthesized a 25-HL photoaffinity probe (**Figure 5**: 25-HL-probe) to identify the binding target of 25-HL and demonstrated that 25-HL binds INSIG proteins, enhances INSIG-SCAP interaction, and blocks SREBP processing, thereby suppressing hepatic lipogenesis.

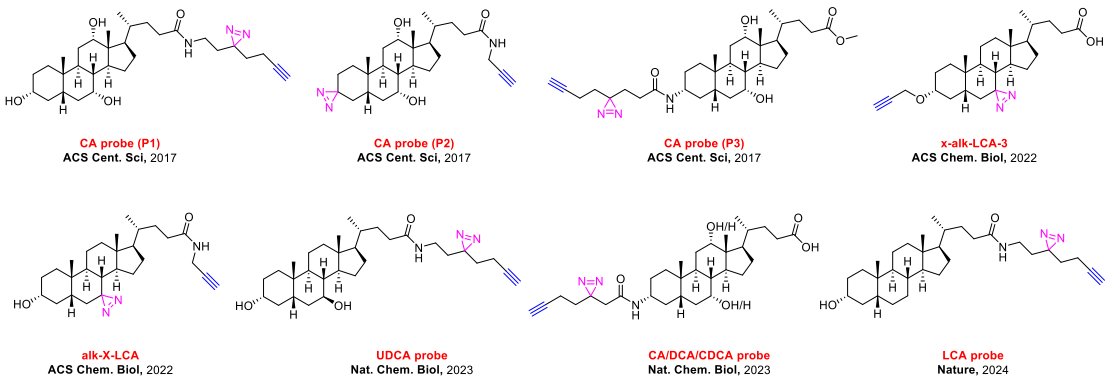

Figure 6. Chemical structure of representative clickable photoaffinity probes designed for different bile acids.

Bile acid probes

Bile acids, originally synthesized in human liver, and metabolized by gut microbiota, serve as critical regulators of host physiology and microbial ecology (Begley *et al.* 2005; Gipson *et al.* 2020). Functioning as amphipathic surfactants, bile salts can disrupt bacterial membrane integrity and compromise cellular homeostasis in intestinal microbiota (Begley *et al.* 2005). Furthermore, emerging evidence indicates that bile acids modulate virulence gene expression and pathogenicity in enteric pathogens (Alavi

et al. 2020; Tam *et al.* 2020). Numerous studies have investigated the regulatory functions of bile acids in shaping the human gut microbiota and elucidating the molecular mechanisms underlying host–microbiota crosstalk.

The Lei lab and the Wang lab (Zhuang *et al.* 2017) pioneered the design and synthesis of bile acid clickable photoaffinity probes. In 2017, they developed a number of photoreactive primary bile acid (BA)-derived molecular probes and systematically identified >600 BA-associated proteins by chemoproteomics. Proteomic analysis revealed significant enrichment of these proteins in critical cellular pathways, particularly endoplasmic reticulum (ER) stress response and lipid metabolic processes. Bioinformatics predictions further suggested strong pathophysiological associations with Alzheimer's disease, non-alcoholic fatty liver disease (NAFLD), and diarrheal disorders. This groundbreaking work substantially advances our mechanistic understanding of BA-mediated regulatory networks in both physiological homeostasis and disease pathogenesis.

Lately, the Lin lab (Qu *et al.* 2024a) elucidates a conserved signaling axis through which lithocholic acid (LCA) activates AMPK via sirtuin-mediated metabolic regulation. Mechanistically, LCA binds TUB-like protein 3 (TULP3) to allosterically activate sirtuins, which deacetylate specific lysine residues (K52/K99/K191) on the V1E1 subunit of v-ATPase. This inhibition of v-ATPase triggers AMPK activation via the lysosomal glucose-sensing pathway. Genetic evidence demonstrates that muscle-specific expression of a deacetylation-mimetic V1E1 (3KR) activates AMPK and rejuvenates aged mouse muscle; and LCA extends lifespan in nematodes and flies via homologous TULP3 partners (tub-1/ktub). The findings establish the TULP3–sirtuin–v-ATPase–AMPK pathway as a molecular bridge connecting LCA signaling to calorie restriction-like benefits across species.

A comparative chemoproteomic analysis of primary and secondary bile acids in mammalian cells revealed that secondary bile acids directly bind to the transmembrane domain of the stimulator of interferon genes (STING) (Yang *et al.* 2025). Interestingly, the secondary bile acid deoxycholic acid (DCA) does not directly activate STING but instead synergizes with other STING agonists to enhance STING activation. This study highlights the value of proteomic approaches in uncovering molecular mechanisms of bioactive metabolites—mechanisms that may be overlooked by conventional functional screening, as DCA alone does not elicit STING activation. This study uncovered an unexpected mode of action for DCA in modulating STING signaling and suggests that

specific secondary bile acids and their associated microbiota may influence the therapeutic efficacy of STING-targeted treatments.

In addition to regulating mammalian cell signaling, bile acids also influence bacterial physiology, particularly that of gut microbiota, which reside in an environment with high concentrations of bile acids. In 2022, the Wang lab and Lei lab (Liu *et al.* 2022) employed a chemoproteomic approach to identify bacterial bile acid (BA) sensors, revealing histidine kinases (HKs) of two-component systems (TCS) as novel BA-binding proteins. Genetic and biochemical validation pinpointed EnvZ as a key BA sensor, where BA binding triggers a detoxification pathway to promote efflux and tolerance. These findings establish EnvZ-mediated TCS signaling as a bacterial BA resistance mechanism, offering potential therapeutic targets against BA-tolerant pathogens. The Shen lab (Forster *et al.* 2022) used chemical proteomics strategy in *C. difficile* to identify the MerR-family transcription factor BapR as a lithocholic acid (LCA)-binding regulator. Biochemical and genetic analyses demonstrated that LCA stabilizes BapR, which directly represses the *mdeA-cd3573* locus encoding sulfur-metabolic enzymes and putative transporters. While *BapR* deletion did not alter LCA susceptibility, it caused cell elongation and *mdeA* derepression in LCA-treated cells, revealing BapR as an LCA-sensing hub coordinating metabolic adaptation to gut environmental cues. The Hang lab (Yang *et al.* 2022a) employed activity-guided chemical proteomics strategy in *Salmonella* Typhimurium to identify chenodeoxycholic acid (CDCA) as a potent inhibitor of virulence gene expression and epithelial invasion. Proteomic analysis revealed direct binding of CDCA to HilD, a master virulence regulator, with biochemical and genetic validation demonstrating CDCA-mediated HilD inhibition as a key mechanism of bile acid resistance. Characterization of bile acid-resistant HilD mutants in infection models established HilD as a critical target for anti-infective bile acids, showcasing chemical proteomics as a powerful tool to elucidate microbiota metabolite-pathogen interactions. In the same year, lithocholic acid (LCA) photoaffinity probe (x-alk-LCA-3) (Yang *et al.* 2022b) was developed to map bile acid-protein interactions in *Enterococcus faecium*. Chemical proteomics identified multiple LCA-binding targets involved in cell wall biosynthesis, transcriptional regulation, and metabolism. Functional validation of a candidate bile salt hydrolase (BSH) confirmed its ability to convert taurolithocholic acid (TLCA) to LCA both in bacterial cultures and in vitro. These findings demonstrate the utility of

activity-based proteomics for uncovering bile acid-mediated mechanisms in gut microbiota.

Conclusion and Perspectives

Clickable photoaffinity probes have revolutionized our ability to systematically dissect sterol and bile acid biology by enabling proteome-wide mapping of metabolite–protein interactions. These powerful tools have uncovered both known and previously unrecognized targets involved in metabolic regulation, immune signaling, neurobiology, and host–microbe interactions. Although follow-up approaches are still required to validate each metabolite's functional impact on its protein partners, the spatial and mechanistic detail provided by photoaffinity probes and chemoproteomics has already broadened our understanding of sterol and bile-acid biology.

Looking ahead, the development of next-generation probes with enhanced selectivity, minimal structural perturbation for other sterols and microbiota generated structurally diverse bile acids will be critical for resolving the complexity of sterol and bile acid signaling across tissues, physiological states and microbe-host interactions. Combining state of art chemoproteomics (such as TMT based quantitive proteomics) with emerging technologies, such as cryo-EM(Cheng 2018), machine learning-based structural modeling (Abramson *et al.* 2024), single cell proteomics, will further deepen our understanding of sterols and bile acids-mediated regulatory networks in health and disease.

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COMPLIANCE WITH ETHICS GUIDELINES

Miaomiao Xu, Xiaoming Chen, Jian Ma and Xinglin Yang declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by the any of the authors.

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