ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 1, pp. 1-20. © Pleiades Publishing, Ltd., 2007.
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REVIEW

Photoaffinity Labeling and Its Application in Structural Biology

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Received September 18, 2006
Revision received October 4, 2006

Abstract—This review contains a brief consideration of some theoretical aspects of photoaffinity (photoreactive) labeling (PAL), and the most widely used photoreactive groups, such as arylazide, benzophenone, and 3-(trifluoromethyl)-3-phenyldiazirine, are characterized in comparison. Experimental methodology is described, including modern approaches of mass spectrometry for analysis of cross-linking products between the photoreactive probes and biomolecules. Examples of PAL application in diverse fields of structural biology during the last five-ten years are presented. Potential drug targets, transport processes, stereochemistry of interaction of G-protein-coupled receptors with ligands, as well as structural changes in nicotinic acetylcholine receptor are considered. Applications of photoaffinity ganglioside and phospholipid probes for studying biological membranes and of nucleotide probes in investigations of replicative and transcriptional complexes, as well as photoaffinity glycoconjugates for detecting carbohydrate-binding proteins are covered. In combination with modern techniques of instrumental analysis and computer-aided modeling, PAL remains the most important approach in studies on the organization of biological systems.

DOI: 10.1134/S0006297907010014

Key words: photoaffinity labeling, ligand-binding sites, photoreactive probes, gangliosides, nucleotides, glycoconjugates

Photoreactive probing (photoaffinity labeling (PAL)) is widely used in structure-function studies of biological systems because this approach allows direct verification of the spatial proximity of molecular components (reviews [1-11]). A probe, which most often is a natural substance analog supplemented with a photoreactive group (label, photophore), is introduced into a system under study and subjected to irradiation. Photolysis of the label results in a highly reactive intermediate capable of covalent binding (cross-linking) with the nearest fragment of a biomolecule. For detection of the cross-linking product, i.e. the macromolecule and, finally, the site(s) of its modification, the probe has to contain a reporter (radioactive, chromophore, fluorescent, or immunoreactive) group. And this enables the detecting of specific amino acid residues of a protein which are in contact with the ligand. To obtain such information, structural biology most often uses X-ray crystallography and high performance NMR approaches which are now supported by technologies of preparation of recombinant DNAs capable of producing milligram quantities of polypeptides or polynucleotides to be studied. However, in many cases such a structural analysis is impossible or extremely difficult. In particular, this is especially true for studies on complexes of transcriptional factors, ribosomes, proteins of vesicular systems, local structures of cytoskeleton, and integral signaling complexes. In such cases, PAL is the most important approach for identification of subunits and mapping the active site, in addition and combination with studies by point mutagenesis and monoclonal antibody binding.

PAL was first proposed more than 40 years ago [12], but now it again attracts attention due to elaboration of more sophisticated photophores [4-8]. The development of more effective methods for separating and detecting fragments of macromolecules and also arising of proteomics make markedly easier structural analysis of PAL

Abbreviations: Bp) benzoylphenyl; Bpa) *p*-benzoylphenylalanine; CRF) corticotropin-releasing factor; DAT) dopamine protein transporter; Dcp) diazocyclopentadien-2-ylcarbonyl; ESI) electrospray ionization; GPCR) G-protein-coupled receptor; GSph) gangliosides; ILBP) ileal lipid-binding protein; LC/MS) liquid chromatography/mass spectrometry; MALDI) matrix assisted laser desorption ionization; MDR) multiple drug resistance; MS) mass spectrometry; MS/MS) tandem mass spectrometry; nAChR) nicotinic acetylcholine receptor; Nab) 2-nitro-5-azidobenzoyl; Nap) 2-nitro-4-azidophenyl; PAL) photoaffinity labeling; PC) phosphatidylcholine; SE) squalene epoxidase; TD) transmembrane domain; Tfd) 3-(trifluoromethyl)-3-phenyldiazirine.

products [13]. Combined with computer-aided molecular modeling, the findings can be used for designing models of ligand–receptor complexes [14]. A relatively new substituted Cys-accessibility scanning method (SCAM) [15] makes possible studies on the interaction of ligands only with water-available fragments of receptors. To overcome this limitation, the method can be supplemented by labeling of the active site with ligands carrying reactive groups [16]. But such an approach is difficult in the case of peptidergic receptors. The main advantage of PAL is its applicability for native proteins.

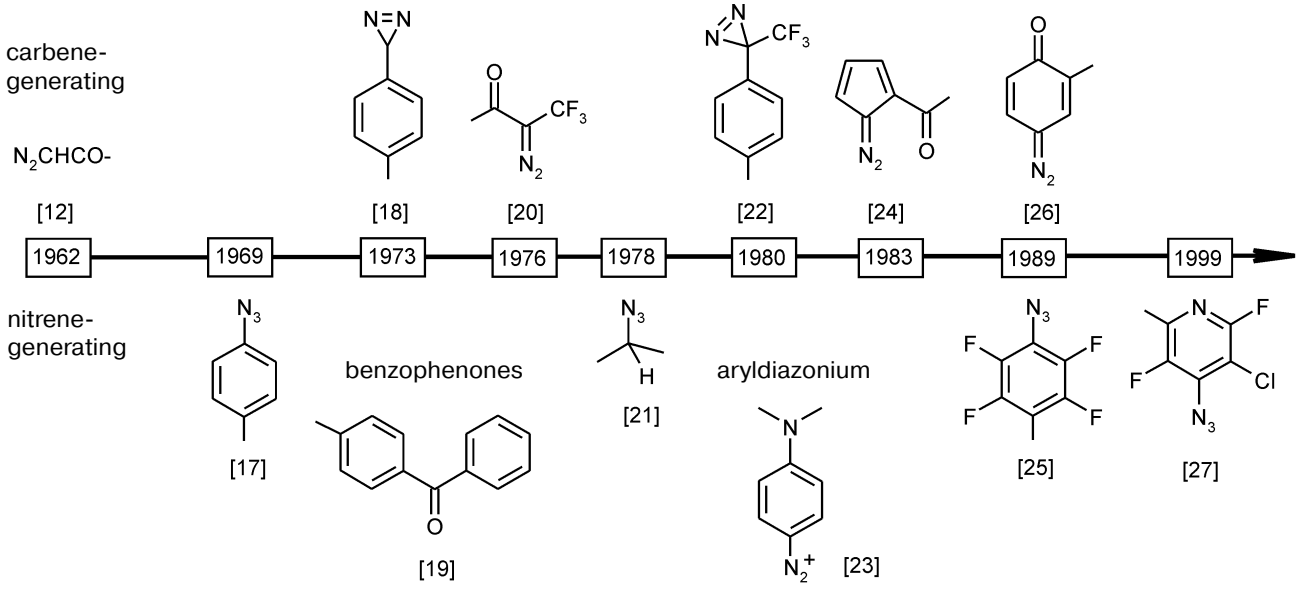
FUNDAMENTALS OF PHOTOAFFINITY PROBING

Photoaffinity probes need to fit the following requirements: the probe must be chemically inert in the absence of actinic light; the photophore has to be activated under mild conditions and its activation must not damage the biosystem and its components; the lifetime of the excited state of the label has to be shorter than the lifetime of a ligand–receptor or another complex under study; the activated probe has to nonspecifically react with any neighboring group, including saturated CH-chains of lipids and nonpolar amino acid residues, with production of a tight covalent bond; the photophore must not induce significant disorders in the biosystem organization; the photophore introduction into the initial substance molecule must not considerably decrease the biological activity; the probe has to contain a radionuclide with a sufficiently high specific activity or an additional label attached through an elongated linker; the probe has to be available.

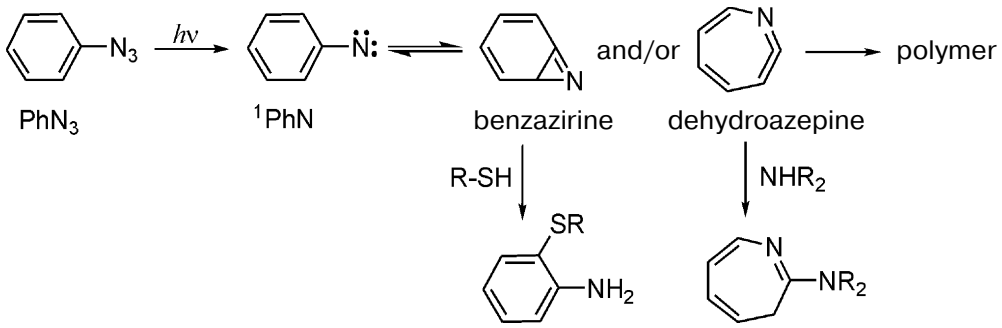
There are no "ideal" photophores fitting all of these requirements. Compounds which on irradiation generate carbenes and nitrenes (excited states of C and N), benzophenone derivatives producing biradicals during photolysis, and also aryldiazonium generating a highly active aryl cation suit these requirements best. After Westheimer's proposal of 1962 [12] to use the diazoacetyl group for PAL, many efforts have been directed to find different photophores (figure), and their properties are still under investigation. We shall briefly consider some features of photochemical reactions.

Absorption of certain wavelength light by a carbene- or nitrene-generating molecule causes its transition into an electron-excited state, which results in detachment of a N_2 molecule and production of a highly reactive uncharged intermediate capable of existing in a singlet (S_1, electron spins are antiparallel) or triplet (T_1, spins are parallel) state [28]. The primary product of photolysis is a zwitterion type S_1-intermediate characterized by electrophilic reactions. The lifetimes of the S_1-states are short (e.g., for phenylnitrene it is ~10^{-9} sec as shown by laser flash photolysis [29]). Transition into the T_1-state characterized by one-electron radical reactions is a competing process. The covalent binding of the singlet with an adjacent molecule is more probable because it attacks the electron pair in one stage. In the case of the triplet, the bond is a result of two consecutive radical reactions; therefore, there is an increased probability of a simple abstraction of hydrogen atoms from the target molecule [30].

Carbenes are more reactive, i.e., less selective with respect to different bonds than nitrenes. Usually S_1-carbenes including phenylcarbenes react very rapidly with production of stable adducts with the majority of func-



Chronology of reports about the application of different functional groups for photoaffinity labeling



Phenylazide transformations under photolysis in solution

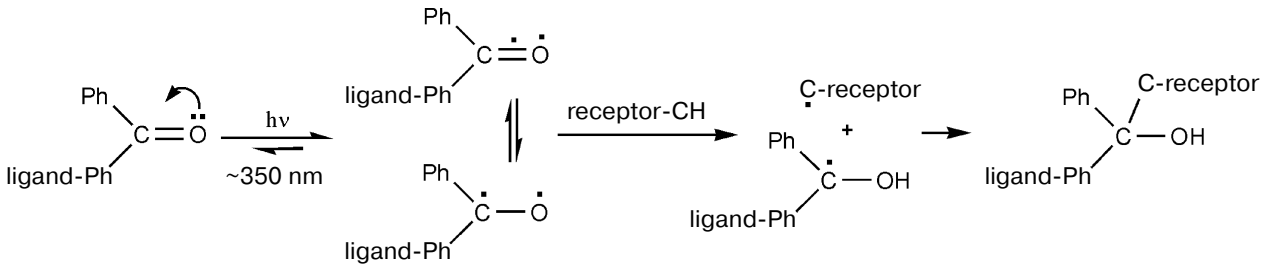
Scheme 1

tional groups (OH, Ar, –C=C–, and even inactive CH-bonds of alkanes): the rate constants of bimolecular reactions are 10^{-7}-10^{-9} $M^{-1}\cdot sec^{-1}$ [31]. Unsubstituted S_1-arylnitrenes rapidly rearrange intramolecularly into long-lived electrophilic intermediates, such as benzazirine and/or cyclic ketenimine dehydroazepine capable of reacting with remote nucleophilic groups [29, 31], which gives false results of PAL (Scheme 1).

A fundamental difference between singlet phenylnitrene and phenylcarbene is confirmed by modern calculations using molecular orbital theory: ^{1}PhN is prone to cyclization because of existence of electron structures with uncompleted envelopes [31]. Obviously, PhN_3-groups have been used for PAL more often than other groups due to commercial availability of synthons for the preparation of probes. Photochemical properties of ^{1}PhN are influenced by the substitutes. Perfluorophenyl nitrenes are more active and not prone to cyclization [25, 31, 32]. Phenylazides can be photolyzed under mild conditions (>300 nm), although their absorption is characterized by λ_{max} < 280 nm and to provide for PAL efficiency long-term irradiation (to 2 h) is needed, which is undesirable in some cases.

Diazirines are characterized by photoisomerization into electrophilic diazo compounds [6]. The 3-(trifluoromethyl)-3-phenyldiazirine (Tfd) group [22] during photolysis (~360 nm) produces more than 30% of diazo compound, which can generate carbene upon the more severe irradiation. Unlike $PhCHN_2$, diazo isomer of Tfd-label displays no tendency for protonation and subsequent attacks by nucleophiles; therefore, side reactions characteristic for phenyldiazirines (e.g. alkylation of COOH-groups) are inhibited [22]. The Tfd-label has important advantages and is a leader in PAL [7]. But the following shortcomings should be noted: complicated scheme of synthesis, possible loss of the labeled substrate upon the introduction into the NH-bond (due to HF detachment and subsequent hydrolysis with production of ketone) [33], and also instability of some PAL products under conditions of proteolysis during peptide mapping [34], which seems to occur on the mapping by OH-bonds of side chains of E, D, and Y [3].

Benzophenone (Bp) groups are often used for PAL [8, 10]. Methods for expression in animal [35] and *E. coli* [36] cells of proteins containing *p*-benzoylphenylalanine (Bpa) have been developed which enable the study of weak interactions of proteins or their intermediate complexes. The popularity of Bp-groups (despite their large volume) is explained by their higher chemical stability, commercial availability, and photochemical properties: being photoactivated at ~350 nm, they react with inactive CH-bonds even in the presence of water (but the photolysis needs more prolonged time, at least 30 min, and is usually performed at 0°C). As distinguished from photolysis of arylazides and diazirines, the generation of biradi-



Photolysis of Bp-labeled ligand and subsequent interaction with receptor

Scheme 2

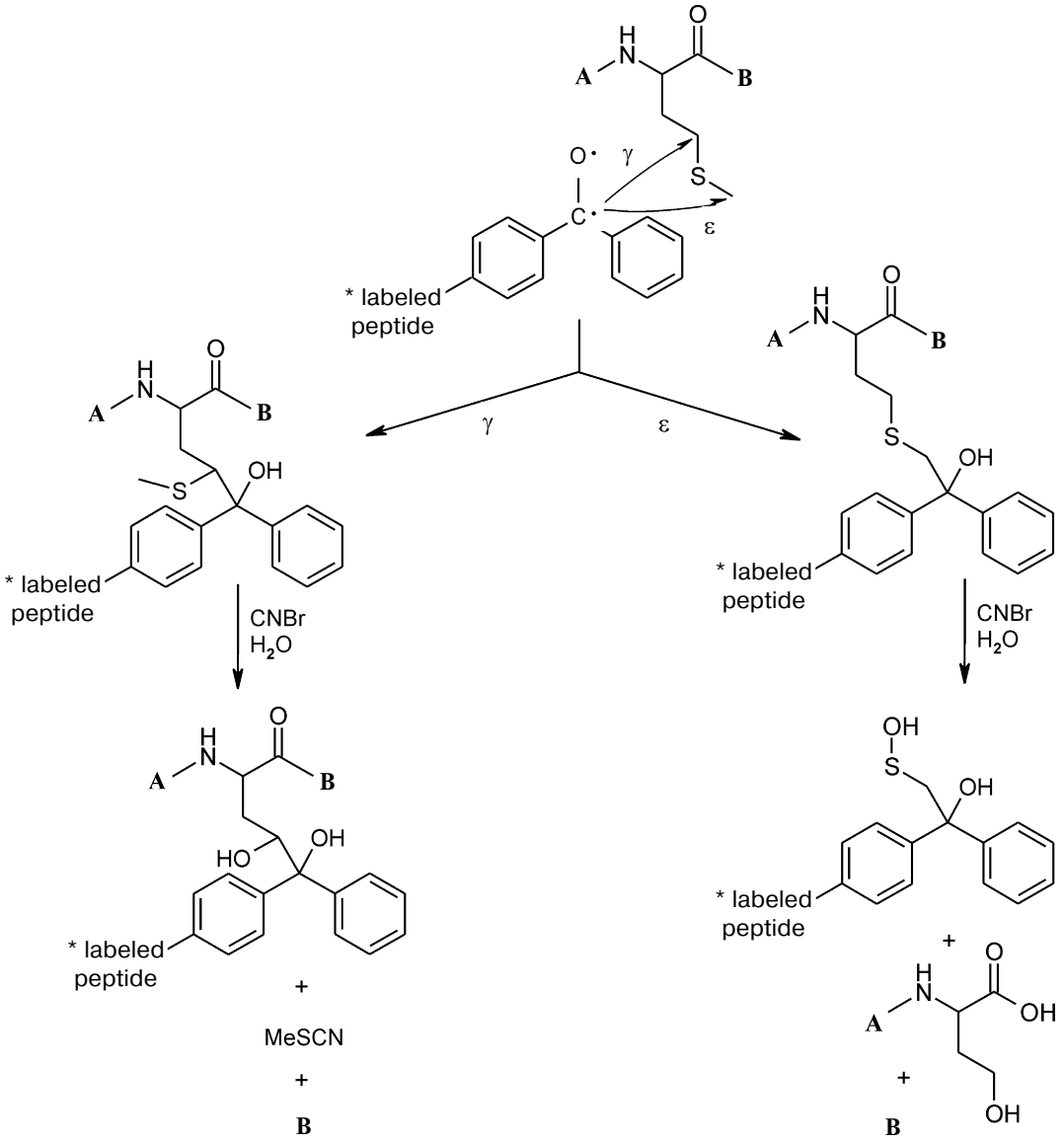
cals from Bp is reversible, and this increases the labeling efficiency of the substrate (receptor) due to multiple excitation–relaxation cycles [8, 10] (Scheme 2).

However, in some physiologically active peptides Bpa was markedly selective with respect to Met residues in the receptors [37-41]; therefore, an original approach was even proposed for scanning the binding sites of peptidergic receptors coupled with the G-protein [42, 43]. This approach was based on a unique feature of Met: the Met residue of the receptor labeled by the thiomethyl group by Bpa-containing peptide released the labeled peptide after the CNBr-cleavage, as shown in the Scheme 3 [37]. The molecular modeling gives the PAL radius of Met residues by the Bpa keto-function to be ~6-7 Å [42] and not 3.1 Å [10].

The comparison of PAL efficiency for peptides [44] and DNA [45] using probes carrying PhN_3, α-diazocarbonyl, Bp-, and Tfd-labels indicated the advantage of the Tfd-label.

SCHEME OF PHOTOAFFINITY LABELING EXPERIMENTS

When constructing the probe, it is desirable to retain its biological activity at the level of the initial compound,



Interaction of Bpa-labeled peptide with receptor Met residue and subsequent CNBr-cleavage. **A** is the N-terminal sequence of the receptor; **B** is the C-terminal sequence of the receptor. * Labeled peptide, the radiolabeled peptide-ligand

Scheme 3

but even 1000-fold less active derivatives can be useful in searching for new drugs [10]. The probe has to carry a radioisotope or another label (e.g., biotin [46, 47]) bound through an elongated linker. Highly active radionuclides (^{125}I, ^{32}P) providing for the highest sensitivity of the method are introduced into the probe immediately before PAL.

The PAL products are identified on three levels [10]. On the *macro level*, proteins or their subunits to which the probe is first of all bound are determined. This information is important for studies on the organization of oligosubunit integral membrane receptors. This information is usually sufficient for biomolecular screening when individual candidates for drugs or large molecular libraries prepared by combinatory organic synthesis are under testing. On this level, the cross-linking products are mainly analyzed by SDS-PAGE; the labeled polypeptides are detected by radioautography or fluorography. Upon detection of the purposeful protein (proteins), the binding specificity is proved: the labeling by the probe has to be suppressed in the presence of 100-1000-fold excess initial ligand [10].

On the *semimicro level*, the specifically labeled proteins are isolated and subjected to enzymatic proteolysis or chemical cleavage at specific amino acid residues, and then separated by electrophoresis or HPLC to detect the binding site on the peptide level. In fact, after the labeled peptides have been isolated, the radiolabel is already not necessary, because the peptides can be analyzed using mass spectrometry approaches (MALDI or ESI) or degradation by the Edman method in the case of sufficient amount of the protein. Moreover, liquid chromatography/mass spectrometry (LC/MS) allows the analyzing of peptide–protein cross-links on both macro- and semimicro levels even without a radiolabel [13, 34]. The identified binding site can be expressed, and the recombinant peptide (peptides) can be used for investigation of ligand–receptor interactions.

On the *micro level*, key amino acid residues of the receptor which are involved in binding with the ligand are conventionally determined using degradation by the Edman method: the labeled peptide is subjected to automated or manual radiosequencing [48, 49]. The photolabeled amino acid residues in peptide fragments are directly determined by tandem mass spectrometry (MS/MS) [50].

USE OF PHOTOREACTIVE LIGANDS FOR ELABORATION OF DRUGS

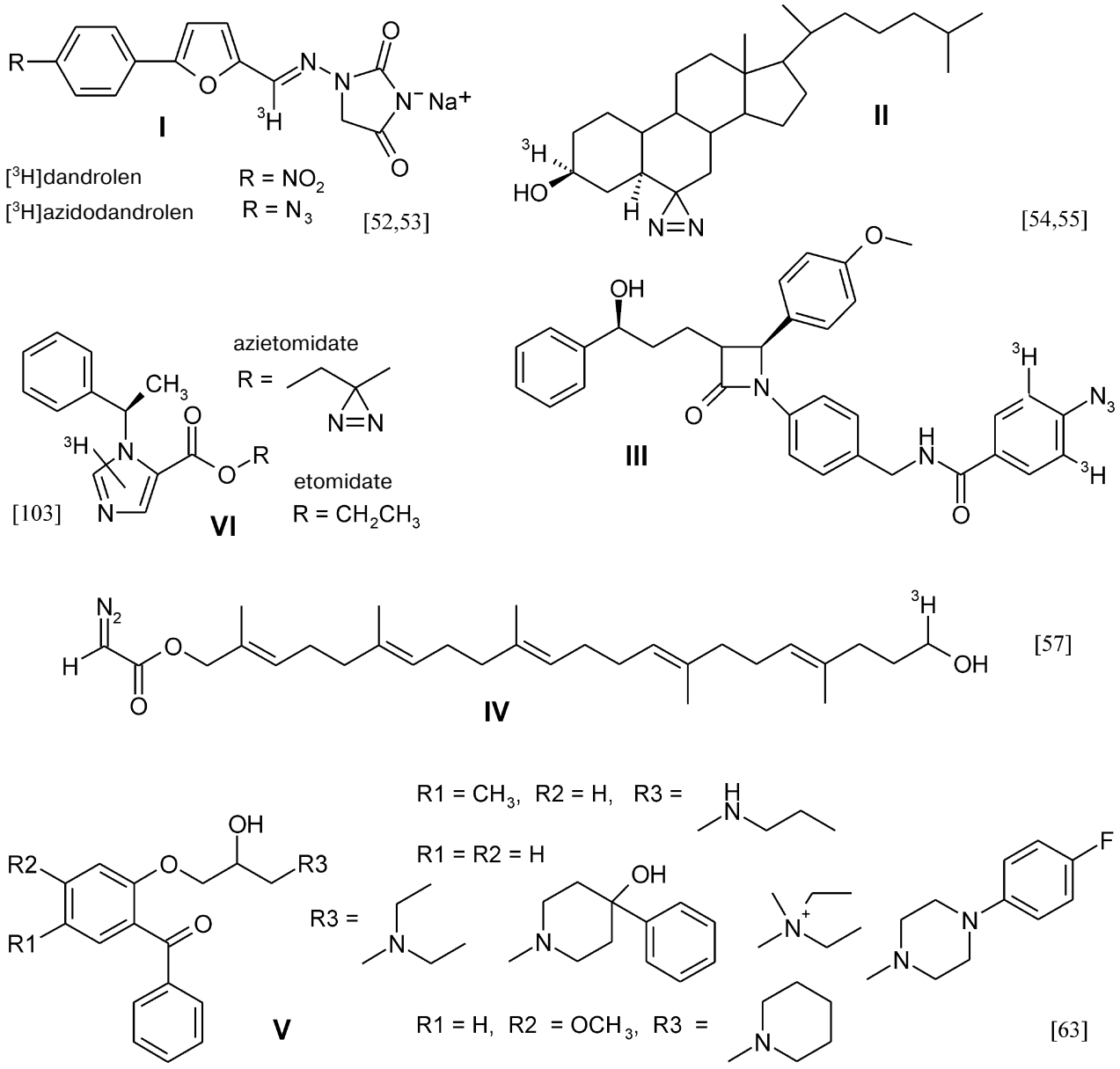
Data on the binding site structure of a purposeful receptor facilitate the development of an optimal pharmacophore. Modern technologies, such as combinatorial chemistry, gene engineering approaches, and high-throughput analysis methods have made PAL a tool for identification of drug/receptor pairs and also for investigation of ligand–receptor interaction mechanisms [11]. In the 1990s various regulatory mechanisms were discovered, including endogenous ligands and their receptors, enzyme–substrate complexes. However, exact targets are still unknown for many drugs, which are on the stage of development or already used in practice. Consider the reports for the last 5-7 years (for earlier years see review [10]).

Search for potential targets of drugs. The first photoaffinity analog of anandamide was synthesized with the N_3-group at the arachidonoyl terminal C-atom and had a high affinity (K_i = 0.9 nM) for the endocannabinoid receptor CB1; this enables study of the binding site of the endogenous ligand involved in CB1 activation [51].

The preparation dandrolen inhibiting the Ca^{2+} release from the sarcoplasmic reticulum (SR) of skeletal muscles is a unique myorelaxant used in multiple hyperthermia—a congenital disorder in the muscular status manifesting itself in response to volatile anesthetics. The drug action mechanism was studied using [^{3}H]azidodandrolen **I** [52] (Scheme 4). A specifically labeled ~160-kD protein was detected by PAL in SR membranes of the skeletal muscles and found by Western blotting to be identical to the ryanodine receptor (RR) of the skeletal muscles (RR1). RRs are the main class of intracellular Ca^{2+}-releasing channels. In subsequent PAL experiments, the binding domain on RR1 was determined, and the heart muscle was shown to contain a receptor isoform (RR2) with an unavailable native conformation of the dandrolen-binding site [53]. This site seems to be a target for dandrolen derivatives, which are promising as drugs for treatment of arrhythmia.

Intestinal absorption plays a major role in cholesterol homeostasis. The photolabeling with a diazirine derivative of [^{3}H]cholesterol **II** and inhibitor of its absorption 2-azetidinone **III** as arylazide (Scheme 4) revealed specific receptors in the small intestine brush border membranes (80- and 145-kD integral membrane proteins, respectively) [54]. The two proteins share the same tissue distribution in the small intestine and none is of the earlier supposed candidates for transporters (SR-BI, ABC G5/ABC, ABC A1). Later the 145-kD receptor of 2-azetidinone was identified as aminopeptidase N (CD13); by PAL the annexin II/caveolin 1 complex was shown not to be a target of 2-azetidinone, as had been supposed on the basis of immunoprecipitation data [55].

The ileal lipid-binding protein (ILBP, a 14-kD membrane protein) is the only physiologically relevant protein capable of binding bile acids in the cytosol of ileocytes. The ILBP binding site was studied in detail using labeling by diazirine 3- and 7-derivatives of [^{3}H]cholyltaurine [56]. By MS MALDI data, C(3) of the substrate was located near the sequence H^{100}-T^{101}-S^{102}. The controversial position 7 was in contact with R^{122}, as shown by chemical modification and NMR (^{15}N- and ^{15}N/^{13}C-



Photoaffinity analogs of drugs and natural compounds used in molecular pharmacology studies

Scheme 4

labeled ILBP). Thus, the steroid core of bile acids is submerged into the ILBP cavity, whereas the negatively charged side chain is near the entrance.

Squalene epoxidase (SE) catalyzes the transformation of squalene to (3S)-2,3-oxidosqualene, which is a key intermediate of acyclic lipid conversion to sterols in plants, fungi, and vertebrates. Inhibition of SE regulates biosynthesis of cholesterol and can also be used in the designing of fungicides [57]. The structure of SE substrates has to fit strict requirements. A competitive inhibitor of SE (K_i = 18.4 μM), [^{3}H]trisnorsqualene alcohol **IV** diazoacetate, has been synthesized (Scheme 4) [57]. Upon proteolysis of photolabeled SE (a 50-kD recombinant) with selective reagents, CNBr and BNPS-scatol, electrophoresis, and radiosequencing of the first 24 amino acid residues of an 8-kD peptide, labeled K^{399}, R^{400}, and D^{407} were identified. The substitution of the above-mentioned charged amino acid residues by voluminous residues P, F, or W (three triple mutants of SE) caused a decrease in both the enzymatic activity and PAL efficiency, and this confirmed the location of the SE substrate-binding site [57].

A direct binding of the Newmann–Pick C1 type protein (NPC1) with cholesterol was found on the living cell level [58]. NPC1 (a 180-kD glycoprotein; 13 transmembrane domains (TD) of which five consecutive ones form

a sterol-sensitive domain (SSD)) plays an important role in vesicular transport of cholesterol and other lipids from late endosomes; disorders in the transport result in accumulation of nonesterified lipids in lysosomes of neurons and neurodegeneration of the central nervous system. Upon photolabeling by [^{3}H]7,7-azocholestane of intact cells expressing NPC1 labeled by various fluorescent proteins (NPC1-FP) and after immunoprecipitation, a ^{3}H-labeled green NPC1-FP displayed as a single band. PAL was strongly inhibited by excess unlabeled sterol and also as a result of SSD-affecting mutations associated with the loss of functional activity. The PAL specificity was also confirmed by other experiments; the totality of the data suggested the low affinity of NPC1 for cholesterol [58].

Functioning of the cellular retinaldehyde-binding protein (CRALBP), a water-soluble 36-kD protein from the pigment epithelium of the eye retina which accepts 11-*cis*-retinol during the isomerization stage of the visual cycle and carries it to 11-*cis*-retinol dehydrogenase, was studied by PAL of CRALBP with 3-diazo-4-keto-11-*cis*-retinal and subsequent topologic analysis by high performance MS [59]. This probe was earlier used for mapping the ligand movement inside rhodopsin in response to photosensitization [60]. Note that the radiolabel was introduced (the bound retinal reduction to retinol by NaB^3H_4) already after PAL [59]. By combination of LC/MS and MS/MS eight amino acid residues (Y^{179}, F^{197}, C^{198}, M^{208}, K^{221}, M^{222}, V^{223}, M^{225}) were identified, four of which had been earlier predicted to exist. Comparison of H/D-exchange and MS of apo-CRALBP and its complex with 11-*cis*-retinal revealed hydrophobicity of the 198-255 sequence (shielded against exchange). On the basis of crystal structures of three homologous lipid-binding proteins, a structural model of the ligand-binding domain was constructed where all labeled amino acid residues covered the binding cavity of the ligand, except M^{208} located on a flexible loop in front of the cavity. In total, 12 amino acid residues interacted with the ligand [59].

The hexapeptide hexarelin is a synthetic peptide stimulating the release of growth hormone during the binding with the receptor coupled with the G-protein. Hexarelin has been shown to bind with CD36 [61], which is a scavenger receptor expressed in various tissues, including the reticular-endothelial system cells. CD36 is involved in endocytosis of oxidized low density lipoproteins (LDL_{ox}). Bpa-labeled hexarelin bound covalently with M^{169} of CD36, as shown by electrophoresis of fragments produced by enzymatic and CNBr-cleavage. Hexarelin is supposed to interfere in the absorption of LDL_{ox} by macrophages, because the LDL_{ox}-binding domain on CD36 corresponds to Q^{155}-K^{183}. This seems to explain the anti-atherosclerotic effect of such synthetic peptides observed on mice with apolipoprotein E deficiency [61].

Transport processes. A drug-binding site has been found in the pump protein LmrA eliminating metabolites and/or xenobiotics from *Lactococcus lactis* cells [62]. LmrA is a member of a large family of membrane transporters, ATP-binding cassettes (ABC); LmrA is responsible for multiple drug resistance (MDR) to the agents with physicochemical properties similar to those of P-glycoprotein (P-gp) substrates. The specific labeling of LmrA in membrane vesicles by [^{125}I]arylazide derivative of Rhodamine-123 was inhibited by various drugs [62], whereas substances neutralizing MDR including cyclosporin A equally inhibited photolabeling of both LmrA and P-protein. After the treatment of labeled LmrA in gel with protease from *S. aureus* V8 a 6.8-kD peptide was identified which contained the (A^{314}-E^{326}) sequence from the fifth and sixth TDs, and this correlated with data of SCAM scanning indicating the sixth TD.

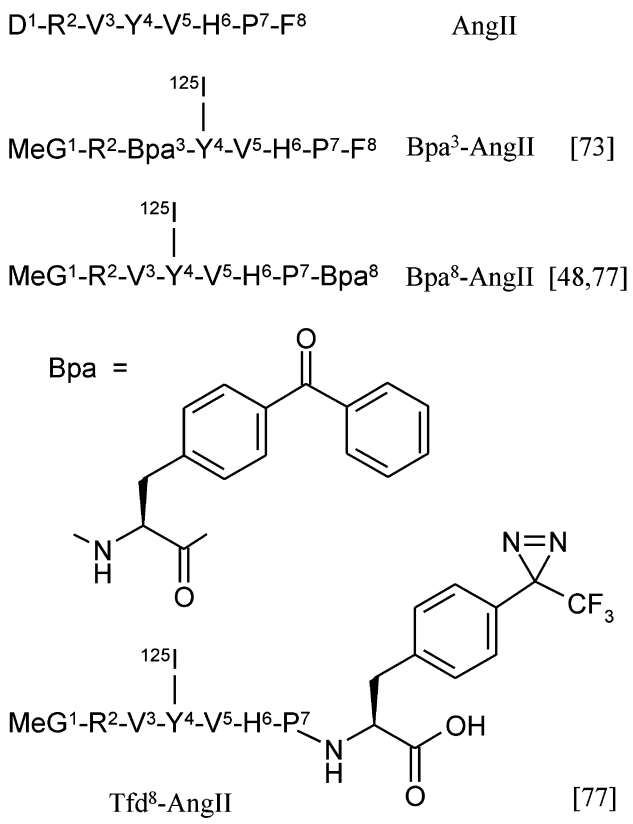
The substrate-binding domain(s) of P-gp were studied using a set of propafenone type (**V**) Bp-analogs [63] (Scheme 4). By MALDI-MS data, the 3rd, 5th, 8th, and 11th TDs were labeled. On the base of a dimeric crystalline structure of the essential lipid MsbA transporter from *Vibrio cholerae*, a homologous model was designed for P-gp using molecular modeling methods, and then the PAL data were projected onto a three-dimensional atomic model of P-gp. The binding was found to occur on the surfaces between two pairs of TDs formed between the N- and C-terminal halves of P-gp: 3/11 TDs from the one side and 5/8 TDs from the other side. Data on two bacterial pump proteins, LmrA and MsbA [63], suggest that the binding of the substrate(s) between the domains should be a distinctive feature for removing transporters with multiple drug specificity.

To identify the cocaine-binding site on the dopamine protein transporter (DAT) in the neuronal membrane, a set of ^{125}I-labeled and nonradioactive arylazide analogs of cocaine and other blockers of the dopamine absorption was used [64]. DAT is composed of 12 TDs, and the N- and C-ends are oriented into the cytosol. The three-dimensional structure of DAT is not resolved; it is unclear how TDs produce conducting pathways for the substrates and where the binding sites of substrate and inhibitors are located. Analysis of proteolytic fragments of photolabeled DAT (cleavage in gel by trypsin, chymotrypsin, protease V8, or CNBr, epitope-specific precipitation) has revealed a 4-kD binding site. More precise localization was established by HPLC of radiolabeled peptides: the retention times were compared with those calculated for the unlabeled peptides and with data obtained for the photolabeled synthetic peptides. By MS MALDI and MS/MS, authentic sequences were detected in the HPLC fractions of the proteolytic DAT fragments photolabeled by nonradioactive ligands. Four of the five ligands bound with TD 1 and TD 2, most conservative for monoamine transporters and, possibly, functionally important for the transport of substrates. Introduction of a variety of struc-

turally different ligands in this area suggests a general mechanism for absorption of blockers and the involvement of these TDs in formation of the binding site(s) and the substrate translocation [64, 65].

Stereochemistry of G-protein-coupled receptor interaction with ligands. The ligand binding sites of the peptidergic G-protein-coupled receptors (GPCR) belonging to a large family of integral membrane glycoproteins similarly built of seven helical TDs are mainly investigated by site-specific mutagenesis. However, changes in the primary structure can influence details of the chimeric receptor architecture and change the ligand behavior in the binding cavity. In the 1990s PAL was successfully used for mapping the ligand binding sites of such class 1 GPCR representatives as the β-adrenergic receptor [66], pituitary receptor of the gonadotropin-releasing hormone (GnRH) [67], adenosine receptor [68], NK1-receptor [69], urotensin 2 receptor [70] (the first three works were performed using ArN_3-labels and the other works with Bpa-labels), etc.

The octapeptide hormone angiotensin II (AngII; Scheme 5), involved in the regulation of many functions of the cardiovascular system, is highly specific to two different mammalian GPCRs, AT_1 and AT_2. Virtually all known functions of AngII are due to its interaction with AT_1, whereas AT_2 is a physiological antagonist of AT_1 involved in the regulation of the central nervous system functions [71]. A model of the AT_1–AngII complex created on the basis of the retrospective analysis of the directed mutagenesis data combined with molecular modeling [72] contradicts the comprehensive PAL studies with [^{125}I]AngII derivatives labeled with Bpa instead of V^3 [73] or F^8 [48] (Scheme 5). The authors [48, 73] proposed a model with an extended conformation of the β-structure of AngII in the binding pocket, its C-terminal amino acid residue contacting F^{293} and N^{294} of the seventh TD of human AT_1, and its third amino acid residue contacting the second extracellular loop (I^{172}). Testing of biological activities of cyclic analogs of AngII confirmed the activity of the extended conformation lacking a bend in the central region of the peptide [74]. In works [48, 73], the labeled peptide fragments were prepared by treatment with a set of proteases (endoproteinases Glu-C, Arg-C, and Lys-C, trypsin), glycopeptidase, and cyanogen bromide in gel, and then were analyzed by manual radiosequencing. Moreover, Met-mutants of AT_1 by the identified contact points with Bpa-AngII probes were used which released the ligand after PAL and treatment with CNBr (see above). The membrane-bound model of AT_1 was constructed [73] arranging the sequences according to work [75] and on taking into account the organization of TDs of the rhodopsin family proteins. AngII is extended virtually parallel to TD in AT_1, with the C-end submerged into the membrane depth and the N-end left on the surface. A similar organization of AngII was shown for its complex with human AT_2, and it is suggested to be specific also for other peptidergic systems [76].



Primary structure of angiotensin II (AngII) and its synthetic ^{125}I-labeled analogs containing Bpa in the third or eighth positions (Bpa^3-AngII or Bpa^8-AngII) or the Tfd-label (Tfd^8-AngII)

Scheme 5

The same authors [42] showed that the Bpa^8-analog of [^{125}I]AngII bound with the M^{128} and M^{138} residues in the third TD of human AT_2 (363 amino acid residues). Therefore, the reactivity and selectivity of two photophores, Bpa and Tfd [77], were compared on the example of AT_2 labeling by Bpa^8- and Tfd^8-analogs of AngII (Scheme 5). The two probes labeled similar CNBr-fragments, but substitution of the Met residues by Ala resulted in a sharp decrease in the Bpa-probe efficiency, as distinct from the Tfd-analog.

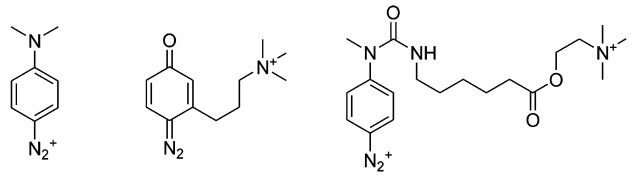
So far PAL has been less frequently used to study the ligand-binding sites of class 2 GPCRs. Bpa-ligands were used in experiments with the receptors of parathyroid hormone [78, 79], secretin [80], calcitonin [81], and with the receptor of the intestinal vasoactive peptide [82, 83] (see the review [84]).

Corticotropin-releasing factor (CRF), a neuroendocrine mediator containing 41 amino acid residues, plays a key role in the regulation of production of adrenocorticotropic hormone and other hormones of the anterior pituitary. Two types of CRF receptors (CRFR) are known which are the class 2 GPCRs: CRF_1R and

CRF_2R. They are distributed in different tissues, mainly in the brain, and are involved in various stress-mediated reactions of the body. CRFRs are attractive pharmacological targets, and the structure of their ligand-binding site has been studied by various indirect approaches using chimeric receptors, deletion-carrying mutants, and mutagenesis [85]. A two-domain model of the CRF binding with CRF_1R has been proposed: the C-terminal of the ligand binds with the extracellular N-terminal of the receptor and forms the main binding domain, whereas the N-terminal of the ligand is slightly submerged into the juxtamembrane domain exposed on the surface and activates the receptor. A three-domain model of binding has been proposed from studies on the interaction of a set of N-terminally shortened urocortins (urocortin is a CRF analog consisting of 40 amino acid residues found in the mammalian midbrain) with the soluble N-terminal region of CRF_1R [86]. This model was tested by PAL of CRF_1R with synthetic analogs of rat ^{125}I-labeled urocortin carrying Bpa in positions 0, 12, 17, or 22 [87]. All analogs had high affinity for CRF_1R and were full agonists of urocortin. The peptide mapping (degradation with proteases, chemical reagents, and endoglycosidase) revealed that amino acid residues 12, 17, and 22 of urocortin were bound not with the N-terminal region of CRF_1R (as believed earlier [86]) but with the juxtamembrane domain. The authors [87] think that the abovementioned amino acid residues are located on the nearest to the membrane side of the ligand helix, whereas intermediate amino acid residues on the other side of the helix are in contact with the N-terminal of the receptor. Thus, urocortin differently binds with the soluble N-terminal fragment of CRF_1R and the whole receptor expressed on the cells. The PAL data have verified the two-domain model and shown that the juxtamembrane domain structures the N-terminal residues of urocortin, which are important for the adequate activation of the receptor [87].

Studies on the structure of the nicotinic type acetylcholine receptor. The current model of molecular organization of nicotinic acetylcholine receptors (nAChR) is designed based on cryoelectron microscopy data [88], directed mutagenesis, and PAL [89]. Muscular and neuronal nAChRs are allosteric cationic channels belonging to the protein superfamily of "Cys-loop" ligand-dependent ion channels, which are activated by an endogenous neurotransmitter acetylcholine (ACh) (review [90]) and play the most important role in synapse functioning. The nAChR functioning is characterized by opening of the channel pore upon ACh binding; the process goes for a few milliseconds and results in a cationic current through the channel and depolarization of the postsynaptic membrane. Then nAChR is desensitized, and this state continues from a few seconds to minutes. The receptor can be in the following conformations: rest (R, the channel is closed), open (A, activated), intermediate (I), and desensitized (D). The nAChRs from the electroorgan of the *Torpedo* electric ray and neuromuscular junctions of vertebrates are pentameric heteromers ($\alpha_2\beta\gamma\delta$) which carry two ACh binding sites in the N-terminal extracellular domain at the distance of ~30 Å above the membrane (at the boundary between the $\alpha\gamma$- and $\alpha\delta$-subunits) and contain cation-selective channel-forming elements inside. Various amino acid residues lining the ACh binding site were determined, and a model with seven binding loops was proposed which was confirmed by a high resolution crystal structure of the ACh-binding protein, the soluble homolog of the ligand-binding nAChR domain [91]. The ACh binding site includes three loops of the major component (the α-subunit) and four loops of the additional component (γ- or δ-subunit). The TD consists of four α-helices (M1-M4): M2 covers the channel wall and the other helices contact the lipid bilayer. The model is consistent with the nAChR structure obtained by cryoelectron microscopy at the resolution of 4 Å [88].

Some examples of the application of PAL for studies on the organization of the ligand-binding site of nAChR are given below. Probes antagonists (such as [^{3}H]DDF) and agonists (such as [^{3}H]DCTA and [^{3}H]AC5) were used:



DDF DCTA AC5

The DDF molecule is an effective photophore and, according to its structure, probes the ammonium residue-binding site; the ACh binding site was shown to consist of at least three loops of the α-subunit [92]. The binding site of the ACh ester function was located using its structural analog [^{3}H]DCTA [93]; based on the dependence of the nAChR (D) photolabeling on the probe concentration, the apparent dissociation constants of PAL of the α- and β-subunits were calculated (2.2 ± 1.1 and 3.6 ± 2.8 μM, respectively). Cyanogen bromide peptides of the labeled α-subunit were separated by HPLC, treated with trypsin, and subjected again to HPLC. The data of automatic microsequencing were processed mathematically, and the labeled amino acid residues were identified univalently (αY^{190}, αC^{192}, αC^{193}, αY^{198}). The same amino acid residues were detected by PAL with the agonist [^{3}H]nicotine [94]. Thus, the ester residue of ACh was shown to interact with the C-loop.

Conformational changes in nAChR on binding with ACh can be studied using time-resolved PAL (review [90]). This method requires large quantities of protein and has been elaborated just for nAChRs, which are produced in abundance by *Torpedo*. The key stage of the method is the fast mixing of samples using a special device before photolysis. Then the reversible ligand–

receptor complex is "quenched" by flash photolysis [95] or fast freezing (–196°C) followed by irradiation in the steady state [96]. The quenching stage determines the time resolution of these approaches (<5 and <1 msec, respectively). The radiolabeling of a protein is measured as the mixing time function: the subunits are separated by PAGE and analyzed for the specific radiolabeling. The subsequent stages of the protein extraction from the membranes, enzymatic hydrolysis, purification of peptides, and microsequencing are used to calculate the radiolabel introduction into the peptides and amino acid residues. A less tedious procedure was proposed on the base of photochemical reactions with the receptor in different states fixed electrophysiologically and subsequent analysis by MS (electrophysiology-coordinated photochemistry (ECP-MS)) [97]. Synchronized voltage pulses (voltage-clamped oocyte) and irradiation enable the labeling of small quantities of nAChRs expressed in oocytes, but with lower time resolution (~500 msec) determined by the buffer exchange in the oocyte-containing chamber. The oocyte membranes are solubilized, and the labeled nAChRs are isolated by electrophoresis, degraded enzymatically, and analyzed by capillary liquid chromatography and ESI MS/MS. However, the ECP-MS method has some limitations. Picomolar amounts of the PAL product preclude quantitative evaluation of the probe introduction. Moreover, usually only a small fraction of proteins is labeled, and this significantly complicates the MS-sequencing and especially the discrimination of the specific and nonspecific labeling. Finally, MS analysis of hydrophobic fragments is difficult, and this seems to explain the failure in detecting the amino acid residues of M1-M3 TDs by photolabeling with hydrophobic probes [97].

Reorganization of the ACh binding site was studied by flash photolysis combined with a stopped-flow setup, at first with a competitive antagonist [^{3}H]DDF [98] (the R $\rightarrow$ D transition), and then with agonists [^{3}H]DCTA [99] (A $\rightarrow$ I $\rightarrow$ D) and [^{3}H]AC5 [100] (A $\rightarrow$ I $\rightarrow$ D). At the receptor transition A $\rightarrow$ I $\rightarrow$ D, the γ-subunit additionally framing the active site moves nearer to amino acid residues Y^{190}, C^{192}, C^{193}, and Y^{198} of the α-subunit C-loop [99]. The probe AC5 has high affinity (K_d = 9 nM) and is a full agonist of nicotine (EC_{50} 1.2 μM) with respect to nAChR of *Torpedo* expressed in the oocytes [100]. As judged from the radiolabel incorporation, the photophore of the probe mainly reacts with the additional components (the γ- and δ-subunits) in state D, and in the transitional states R/A/I – with the α- and γ-subunits. The docking of AC5 (on the model of the ACh-binding protein) indicated that the steady PAL of the α-subunit was possible only in the case of the more open ACh binding site. The probe induced the desensitization and subsequent narrowing of the binding site (the AC5 photophore came nearer to the additional subunit), i.e., a tight package was formed corresponding to the steady state with a high affinity for agonists [100]. The authors of work [90] think that the use of the AC5 probe will facilitate the choice between the two models of the nAChR allosteric transitions, concerted and sequential, which are under discussion.

Studies on the interaction of various nAChRs with powerful blockers, α-neurotoxins (α-NT) from snake venom represent a special line in pharmacology. There is a model of the short-chain α-NTII complex with the extracellular domain of nAChR [101] based on the ^{1}H-NMR structures of α-NTII and data on the interaction of nAChR with α-NTII and homologous short α-NTs obtained by labeling (spin, fluorescent, photoaffinity) and mutagenesis. The main test at the docking was the assessment of mobilities of the whole α-NT molecule and the side chains of Lys with or without photophores; at the final stage, the models were reanalyzed and compared with the experimental data. The results of some experiments with PAL can be explained in the framework of this model (e.g., cross-linking of the nAChR δ-subunit with nitrodiazirine labeled K^{25} and K^{44} [102]).

The majority of general purpose anesthetics act via modulation of responses of ligand-dependent ion channels, in particular, nAChR. The binding site of etomidate, which is one of the strongest intravenous anesthetics used in medicine, was identified by PAL of nAChR with [^{3}H]azietomidate **VI** (Scheme 4) [103]. The sequencing of the enzymatic hydrolysis fragments revealed labeled amino acid residues in the α- and δ-subunits of both the ion channel domain and the ACh binding site. The intensity and distribution of the cross-links depended on the state of nAChR (D or R).

PHOTOAFFINITY LIPID PROBES IN MEMBRANE STUDIES

PAL with lipid probes in membranes and other lipid–protein complexes is characterized by the absence of highly specific lipid–protein interactions similar to ligand–receptor interactions. However, there are multiple contacts with lipid molecules, which form the matrix of these systems. As a result, the probes are mainly spent for cross-linking with lipids that leads to generation of polymeric products (in particular, due to secondary radical processes), which are difficult to separate on analyzing lipid–protein cross-links. Difficulties in analysis of hydrophobic products by MS were already mentioned. Nevertheless, a great deal of valuable information concerning the organization of membrane systems has been obtained by PAL (reviews [3, 4, 9]).

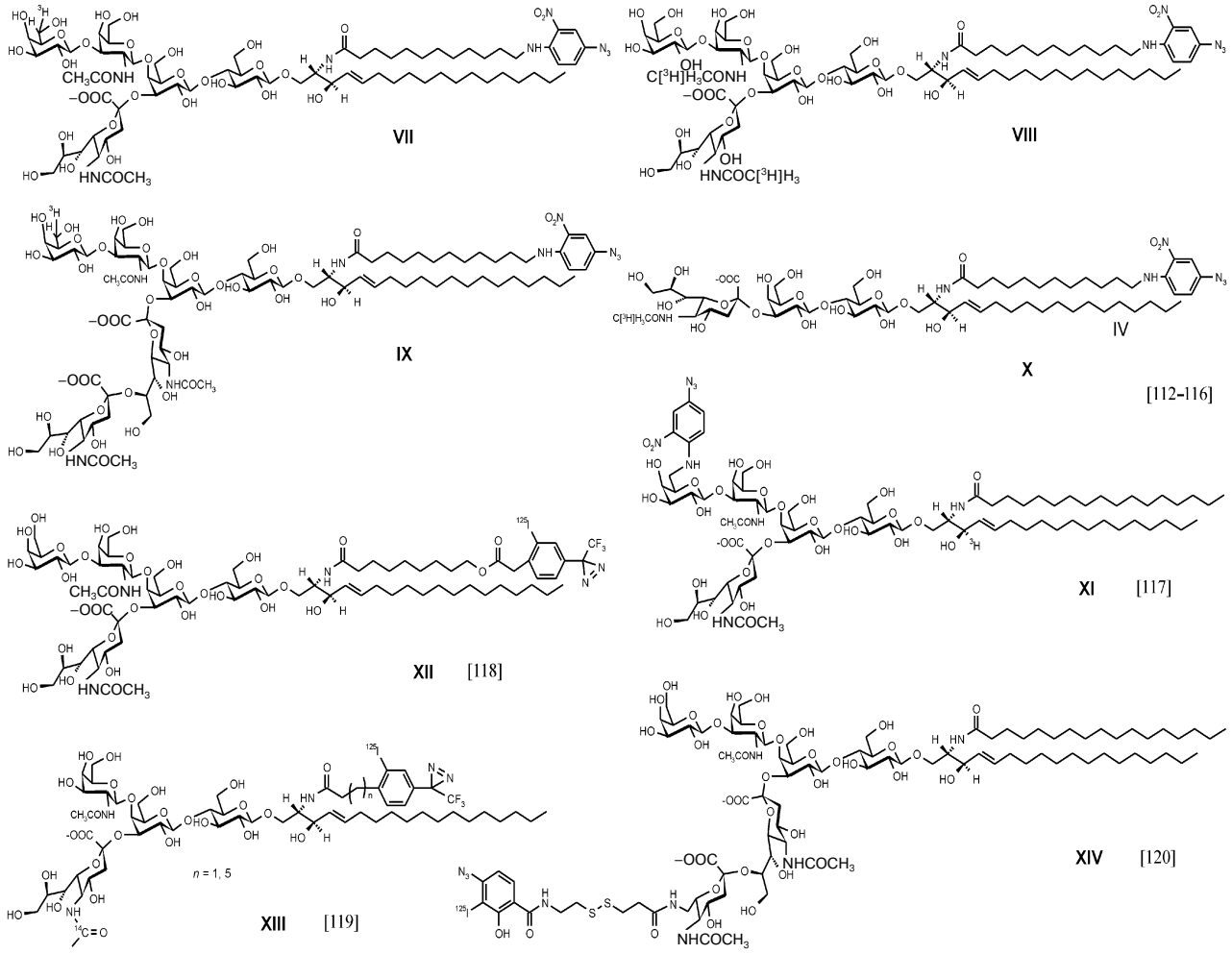
MS of the cross-linking products of phospholipid probes carrying photophores in different positions of the acyl chains [104] reveals the maximum binding in the model membrane with methylene which is three C-atoms shifted towards the polar head of the phospholipid com-

pared to the photophore position. This corresponds to lipid bilayer packing at temperatures higher than that of the phase transition.

Photoaffinity ganglioside probes. Gangliosides (GSph) are glycosphingolipids that carry one or several sialic acid residues (obligatory components of the plasma membranes of eukaryotes) and are especially abundant in the nervous tissue [105]. GSph are located on the outer side of the bilayer exposing oligosaccharide determinants on the cell surface. GSph play an important role in intercellular recognition, modulate cell growth and differentiation, function as receptors, and are involved in information transmission (reviews [106-108]) and apoptosis [109]. The carbohydrate moiety of the GSph molecule is determining in such interactions, but probes with the photophore in the nonpolar (ceramide) moiety allow investigation of membrane rearrangements caused by ligand binding with the receptor where GSph either binds immediately with the protein, or acts as a cofactor. The label introduction into the ceramide moiety does not disturb the structure of the carbohydrate residue, and the probe retains the specificity of the initial GSph.

In the plasma membrane, glycolipids are mainly located in discrete domains, the so-called rafts, which are enriched with sphingomyelin, cholesterol, phosphatidylcholine with saturated acyl chains, and a number of functionally associated proteins (reviews [110, 111]). Rafts are postulated to be involved in the mechanisms of signal transduction, cell adhesion, and lipid–protein sorting; the ganglioside GM1 serves as a marker of the rafts [110]. PAL of caveolin VIP21 (the major protein of caveolae) in kidney and tumor cells with the [^{3}H]GM1-probe **VII** carrying 2-nitro-4-azidophenyl (Nap) in the fatty acid ω-position (Scheme 6) was the first direct proof of glycolipid–protein interactions inside the membrane domains [112].

Schemes for syntheses of GSph-probes with the Nap amino group in the ω-position of the C_{12}-residue and ^{3}H in the carbohydrate Ac-groups of GM1 **VIII** and GM3 **X** or at C-6 of the terminal Gal in GM1 **VII** and GD1b **IX** (Scheme 6) have been reported [113]. Probe **VIII** was first



Photoaffinity ganglioside probes

Scheme 6

used for determination of specific proteins implicated in the incorporation of exogenous GSph in human fibroblasts [114]. The probes **VII-X** labeled tyrosine kinase Lyn from the Src family, a myristoylated protein bound with the membrane from the cytoplasmic side [115], and also the cell adhesion protein TAG-1 [116], which is the major protein in neuronal rafts (it is involved in pathfinding of axons). TAG-1 is bound with the outer monolayer of the cell membrane through glycosyl phosphatidylinositol (GPI). Thus, GSph in the membrane depth are in contact with hydrophobic anchors of the above-mentioned proteins through the terminal parts of the ceramide residues. Because PAL of TAG-1 was similar for probes with different carbohydrate residues (GM3, GM1, GD1b) [116], a question arose whether the polar heads of GSph are involved in the interaction with the extracellular part of TAG-1. After PAL of neurons with the GM1 **XI** probe bearing the Nap-photophore at C-6 in the terminal Gal and ^{3}H in the sphingosine residue (Scheme 6) [117], the cell surface was biotinylated and the rafts were isolated. Of the radiolabeled 135- and 35-kD proteins prepared by immunoprecipitation, the first was immunologically identical to TAG-1. Thus, the GM1 head contacted in the rafts with the exoplasmic domain of TAG-1 or carbohydrate residue of the GPI-anchor [117].

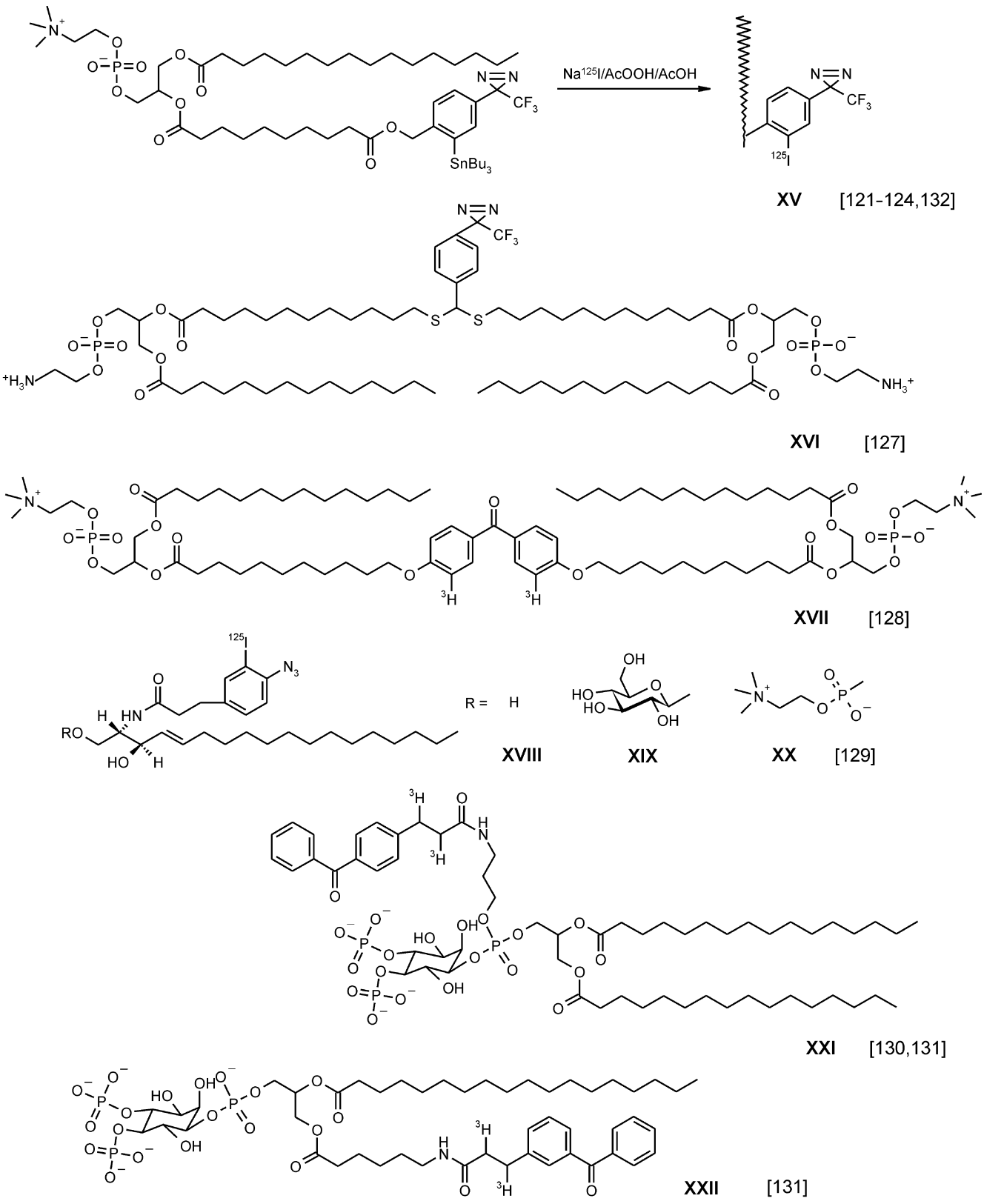
PAL of intact cultured neurons with especially high level of GSph was performed using the GM1-probe with the [^{125}I]Tfd-label **XII** (Scheme 6) under conditions excluding endocytosis [118]. By two-dimensional gel electrophoresis, a 55-kD protein was revealed with pI ~ 5, which carried a considerable fraction of the radioactivity of the raft proteins; this protein was identified as tubulin by immunoprecipitation and western blotting. Because radioactivity of the labeled protein was lost during saponification, the probe was concluded to be cross-linked with the palmitoyl membrane anchor of tubulin. The absence of PAL with the phosphatidylcholine probe bearing the same residue of Tfd-labeled fatty acid confirmed the specificity of the tubulin binding with GM1. Tubulin is known to be involved in signal transduction mediated by G-proteins, and the rafts are shown to contain G-proteins. It seems that the interaction of these two protein families in the rafts influences the signal transduction, and the GM1 interactions with tubulin modulate this process [118].

PAL of the activator protein of GSph GM2 degradation by lysosomal β-hexosaminidase A was performed using Tfd-analogs of GM2 which carried the photophore in the ceramide residue at different distances from the polar moiety and ^{14}C in the NAc-group of sialic acid **XIII** (Scheme 6) [119]. The tryptic hydrolyzate was separated by HPLC, and the resulting radioactive peptides were analyzed by MS MALDI and sequenced by MS ESI. The two probes were specifically cross-linked with the ten-member hydrophobic peptide V^{153}-L^{163}, shown by X-ray crystallography to be the most mobile. The activator protein was concluded to interact with GM2 through this peptide extracting GSph from the membrane and presenting its carbohydrate moiety into the active site of the enzyme. The authors emphasize that MS measurements must be performed accurately [119].

The GSph-binding site of the tetanotoxin C-fragment was identified using the GD1b-probe **XIV** (Scheme 6) labeled with [^{125}I]arylazide by the carbohydrate moiety [120]. The glycerol part of the terminal sialic acid did not affect the toxin reception; therefore, it was subjected to modification. The proteolysis fragments were analyzed by MS MALDI.

Photoaffinity phospholipid probes. Phosphatidylcholine (PC) is the major phospholipid of cell membranes of eukaryotes. The PAL sensitivity of proteins in the lipid bilayer can be enhanced by introducing highly active nuclides into the probe. A whole series of studies was carried out after elaboration of synthesis of the PC analog with the ($SnBu_3$)Tfd-label (Scheme 7), which could be easily radio-iodinated producing probe **XV** with very high specific radioactivity [121]. Glycoproteins from the envelopes of rabies and vesicular stomatitis viruses were found to contain domains interacting at low pH with hydrophobic regions of model membranes and, putatively, acting as fusion peptides; differences in the behaviors of these peptides and the fusion peptide of influenza virion suggested a difference in the fusion mechanisms [122]. The fusion peptide of the influenza virion located in the N-terminal domain of the membrane-bound hemagglutinin HA_2 subunit and exposed for penetration into the cell membrane was shown to be a very small fragment (1-22 amino acid residues) [123]. PC presenting to 50% of mitochondrial membrane phospholipids was supposed [124] to specifically interact with proteins. In yeast mitochondria, probe **XV** specifically labeled a 70-kD protein; upon the isolation by two-dimensional gel electrophoresis and MS of the tryptic hydrolyzate, this protein was identified as a product of the gene *gut2* (60% correlation in peptide mapping), the FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase. (It is interesting that the sequencing revealed an N-terminal sequence of six amino acid residues different from the predicted one, and the authors introduced changes into the database of yeast proteins.) The yeast line with the *gut2* deletions was not specifically labeled. PAL by probe **XV** was observed only under conditions of availability of the inner mitochondrial membrane, along with the parallel enhancement of PAL of the P_i carrier protein (the inner membrane marker). The enzyme was assigned to peripheral proteins because it was extracted from the membrane on destruction of hydrophobic but not electrostatic interactions. The interaction with the inner mitochondrial membrane is thought to be associated with regulation the activity of this protein [124].

The diazocyclopentadien-2-ylcarbonyl (Dcp) group proposed earlier as an effective carbene-generating label



Photoaffinity phospho- and sphingolipid probes

Scheme 7

[24] was found to be easily radio-iodinated with retention of photochemical properties [125]. The Dcp group was comparable with the Tfd label in the ability to insert into the CH-bond of cyclohexane during photolysis; the PC-probe carrying the [^{125}I]Dcp-label in the ω-position of the acyl chain selectively labeled the HA_2-subunit in the influenza virion membrane [125]. This probe was used to study topography of the oligosubunit membrane sector F_0 H^+-ATPase of the mitochondrial inner membrane. In the proteoliposomes containing the active enzyme, a- and c-subunits contacted with lipids (data of electrophoresis and MS MALDI) [126].

Bipolar probes **XVI** [127] and **XVII** [128] were used for precise probing of the central region of membranes (Scheme 7). The phosphatidylethanolamine analog **XVI** was easily introduced into the lipid bilayer without affecting its packing (as shown by electron microscopy); the transmembrane location was verified by reagents for amino groups. The probe efficiently labeled gramicidin A in proteoliposomes, as well as hemagglutinin (the HA_2-chain) of the influenza virus during the virion fusion with liposomes carrying [^{3}H]**XVI** initiated at pH 5 [127]. The integral protein TD center was selectively modified for the first time with PC-probe **XVII**: the label was introduced into V^{80} and M^{81} of glycophorin A (131 amino acid residues) incorporated into liposomes [128].

Arylazide analogs of sphingolipids, ceramide **XVIII**, glucosylceramide **XIX**, and sphingomyelin **XX** (Scheme 7) were used for monitoring lipid transport in mammalian cells [129]. Probe **XVIII** was included in the metabolism with production of the corresponding sphingolipids. Electrophoresis showed that the sorting and metabolism of sphingolipids occurred with involvement of different protein pools depending on the lipid and cell type. Sphingomyelin-labeled proteins were mainly found in the rafts of the cytoplasmic membrane.

A large collection of works was performed using PtdIns-Bp-probes (Scheme 7) to study mechanisms of signal transduction, vesicular traffic, and endo- and exocytosis [10]. Thus, 14-kD human profilin regulated rearrangements in the cytoskeleton of actin. Probe **XXI** photophore reacted with A^1 in the N-terminal helix of profilin [130]; molecular modeling with consideration of the maximal extension of the probe linker indicated that the C-terminal helix amino acid residues R^{135} and R^{136} electrostatically interacted with the 4,5-biphosphate residue. With probes **XXI** and **XXII** it was established [131] that phospholipase C isoenzyme δ1, as opposed to other isoforms, has a substrate-contacting hydrophobic region which is unlike the active site responsible for the enzyme binding with PtdIns-enriched membranes; this region is homologous to plextrin (the so-called plextrin-homologous domains were found in various polypeptides involved in cell signaling and formation of cytoskeleton).

Contents of annular lipids in membrane proteins were proposed to be analyzed by mathematical approximation of the dependence of the probe **XV** (Scheme 7) linking on the basis PC concentration [132]. As differentiated from EPR, this approach does not require large amounts of protein and can be used for oligomeric proteins. The stoichiometry of lipid–protein interactions in model proteoliposomes was determined for three ATPases of the P-type family: 17 PC molecules for the plasma membrane Ca^{2+}-pump, 18 for the sarcoplasmic reticulum Ca^{2+}-pump, and 24 and 5.6 PC molecules for the α- and β-subunits of Na^+,K^+-ATPase, respectively [132].

PHOTOAFFINITY NUCLEOTIDE PROBES

Protein–nucleic interactions are studied using dNTP analogs containing a photophore bound by different length linkers with a heterocyclic base. The analogs are introduced into the oligonucleotide with [5′-^{32}P]primer using DNA polymerases. On irradiation, the primer covalently binds with DNA polymerase (classic labeling). Moreover, upon cross-linking, dNTP analogs can be substrates in the reaction of primer elongation in the presence of template (catalytic labeling). Thus, the interaction of the transcriptional factor TFIIIB with DNA was studied using dTTP analogs containing 4-azidobenzoyl bound through linkers with the fifth position of uracil [133]. Data on the dNTP-binding sites of DNA polymerases obtained through studies on replicative complexes using photoaffinity primers are presented in review [134].

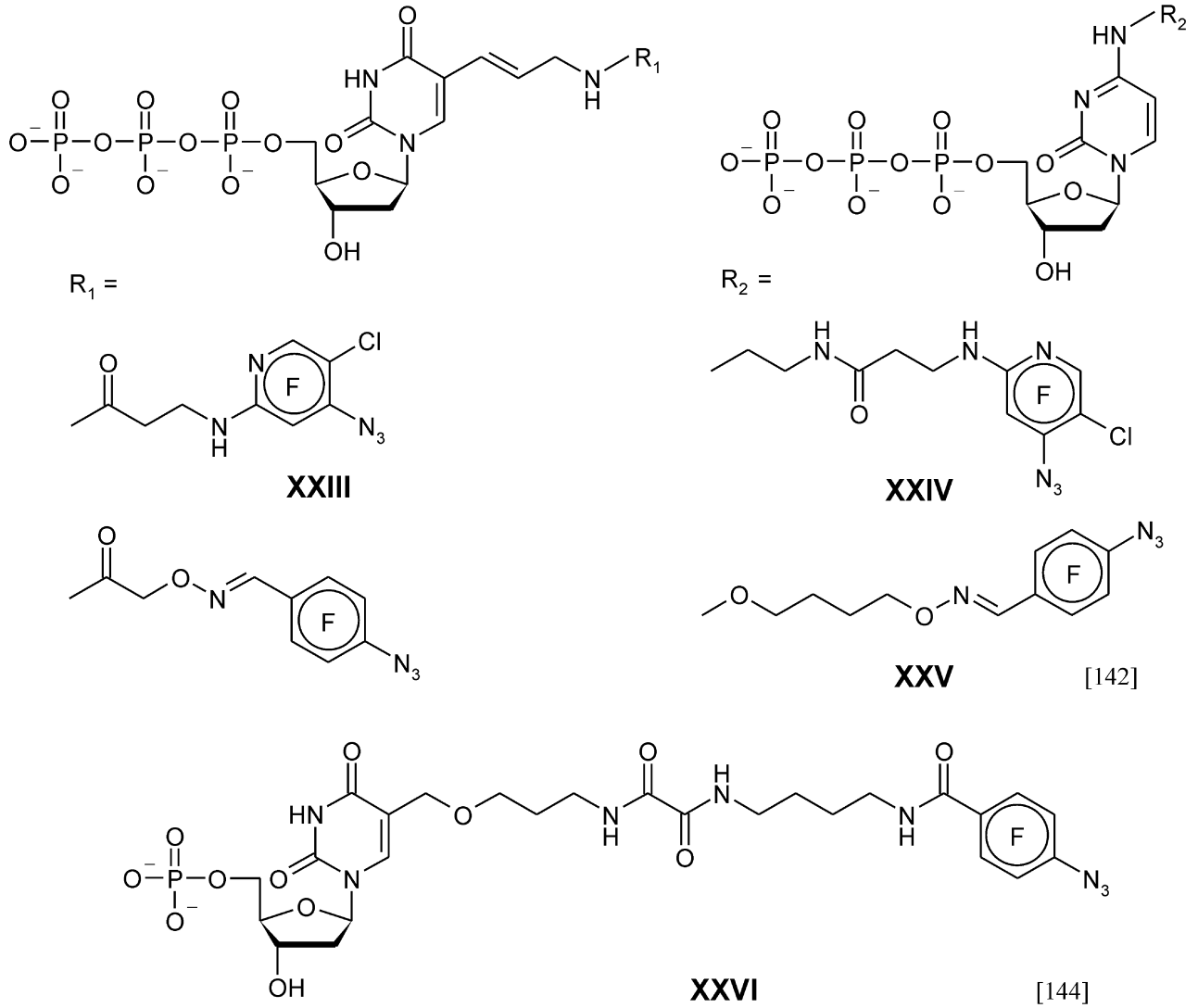
On introduction of the 2-nitro-5-azidobenzoyl (Nab) group into the fourth position of dUTP, the nucleotide retained its substrate properties with respect to HIV-1 reverse transcriptase [135], eukaryotic DNA polymerases α and β, and also the Klenow fragment of *E. coli* DNA polymerase I [136]. Some dUTP-probes carrying the Nab group connected by different length linkers with the fifth position of uracil (Nab-*x*-dUTP, where *x* is the number of atoms in the linker chain) were used to study interactions of DNA-polymerase β and subunits of the human heterotrimeric replicative protein A (RPA) with partial DNA-duplex containing 5′-terminal tip of the primer chain [137-139]. The substrate efficiency of the Nab-*x*-dUTP probes (x = 2, 4, 7-13) relative to thermostable DNA polymerase increased with increase in the linker length [140]; this enzyme is interesting as an object for studies on the accuracy of DNA replication; the introduction of analogs into the 3′-end of the primer did not inhibit the further growth of the chain in the presence of natural dNTPs.

A high sensitivity of PAL is needed for detection of proteins involved in the recognition and repair of damaged regions of DNA in cell extracts. The efficiency of cross-linking with proteins significantly depended on the photophore nature and its position in the heterocyclic

base [141, 142]; perfluoroarylazide-carrying probes were used (Scheme 8). The interaction of excision repair proteins with DNA-intermediates synthesized *in situ* with involvement of endogenous DNA polymerase in extract of mouse embryo fibroblasts was studied using a dCTP-probe containing 4-azidotetrafluorobenzoyl bound with an *exo*-amino group [141]; but PAL was mainly targeted at the complementary chain of the template. The primer containing the dUTP-probe **XXIII** (Scheme 8) with 4-azido-2,5-difluoro-3-chloropyridyl group modified DNA polymerase β with the best yield; therefore, the authors used the dCTP-probe with this photophore **XXIV** as a substrate for endogenous DNA polymerase in extract from HeLa cells [142]; the PAL level of the proteins strongly depended on the efficiency of photoreactive DNA synthesis.

Human apurine/apyrimidine endonuclease 1 (APE1) is one of main components of the excision repair system of DNA bases which hydrolyzes DNA from the 5′-side from the damaged site; APE1 has also 3′-5′-exonuclease activity which is increased to mispaired nucleotides and corrects DNA synthesis during the repair. Conditions for using dCMP-probes for PAL in reconstructed systems and cellular/nuclear homogenates are established: the dCMP-analog of probe **XXIV** is relatively resistant to exonuclease degradation and can be used in APE1-containing systems, contrasting to the probe **XXV** analog (Scheme 8) [143].

The interaction was studied between the proteins involved in the excision repair of nucleotides (RPA and *Xeroderma pigmentosum* of the complementary group A (XPA)) and DNA-duplexes containing bulky pho-



Photoaffinity analogs of nucleotides

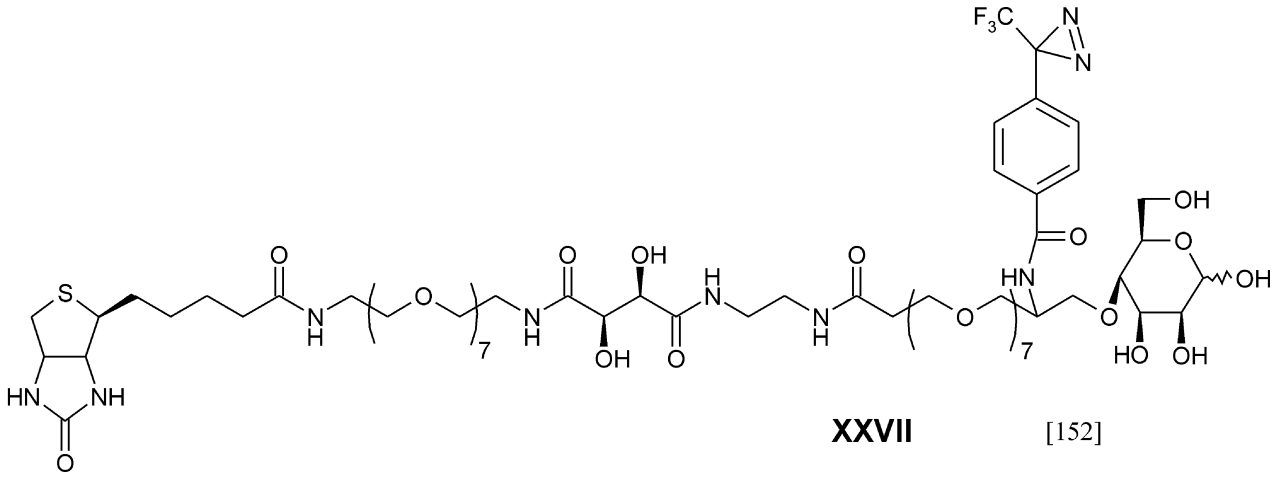
Scheme 8

tophore-carrying substitutes [144]. Probe **XXVI** or dUMP-analogs of probes **XXIII** and **XXIV** (Scheme 8) were linked to the 3′- or 5′-terminal nucleotides in the single-strand nick; the primary recognition of damages, which is the key stage of recognition, was under study. RPA and XPA differed in the sensitivity to the resulting structures. Their roles were discussed from the standpoint of the model of cooperative binding of proteins with nicks.

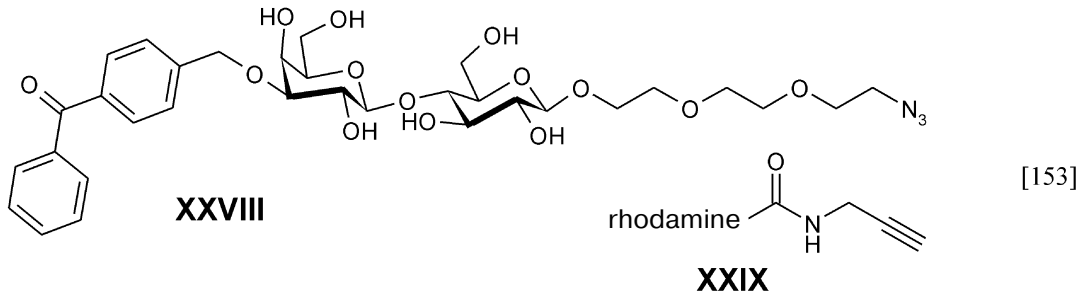
PAL is also used to study functions of the transcriptional complex. Introduction of the ArN_3-photophore by γ-phosphate does not abolish the affinity binding of NTPs. This underlies an approach for modification of RNA polymerase [145]: the reaction mixture containing the template and enzyme is supplemented with the NTP-probe and one or two radioactive NTPs; the synthesized transcript contains the photophore on the 5′-end and irradiation results in covalent binding with RNA polymerase. Thus, the "yield corridor" of RNA can be studied, i.e., the "footprint" of the product replacement along the enzyme with its moving off the catalytic site where the biosynthesis occurs. During the irradiation, *p*-azidoaniline derivatives produce electrophilic derivatives of *p*-benzoquinonediimine [146] capable of selective reacting with amino acid residues; however, if the photophore is bound immediately with γ-phosphate, the reagent can be split from the oligonucleotide in the course of modification. The bond is not broken if the photophore is bound with γ-phosphate-NH-$(CH_2)_n$-NH-spacer (n = 2, 4, 6) [147]. Some probes are synthesized with different photophores (4-azidophenyl, Nab, and 4-azidotetrafluorobenzoyl) and spacer size (n = 2, 3, 4) [148]. The phosphoroamidate bond in the perfluorinated ATP analog can be hydrolyzed, as differentiated from the other derivatives. Unperfluorinated ATP-probes are substrates of RNA polymerase II, but their functional fragments during the irradiation generate long-lived intermediates: *p*-benzoquinonediimine derivatives or nitrosocompounds [148].

PHOTOAFFINITY GLYCOCONJUGATES

In some works associated with the synthesis and application of photoaffinity glycoconjugates for studies on interactions of carbohydrates with proteins (first of all, with lectins which are involved in many cell processes implicated in recognition mechanisms (review [149])), products of PAL were detected by immunoreactive methods or by approaches based on the biotin/avidin system. This made easier tedious purification procedures and allowed the researchers to isolate sought products from



XXVII [152]



XXVIII [153]

XXIX

Photoaffinity glycoconjugates

Scheme 9

sophisticated biological systems, including intact cells. Another specific feature of carbohydrate probe designing is determined by the low affinity for proteins of carbohydrates as they are; therefore, it is more reasonable to bind the photophore to the sugar residue not directly but through a spacer. (High affinity interactions with lectins are provided for by multiple point contacts during the generation of carbohydrate clusters by dendritic oligosaccharides or flexible polymeric glycoconjugates [149].)

A new 33-kD lectin produced in rat cell nuclei in response to stress was identified using an α-D-Glc conjugate with lysyl-lysine labeled by *p*-azidobenzoyl and a voluminous digoxin residue as a reporter group (detection by Western blotting) [150]. Later, on constructing the probes the authors [151] introduced carbohydrate determinants which provided for multiple point contacts: Man_9 or a synthetic neoglycopeptide with three terminal residues GalNAcβ were bound with Lys-Lys labeled by the Nap-group and digoxin. A mannose-binding protein was detected in bovine serum, the binding subunits of the asialoglycoproteins were identified in the plasma membranes of rat hepatocytes, and the monitoring of lectins was performed in human sera during the progression of juvenile chronic arthritis [151]. However, the authors warn about the danger of artifacts, such as a specific binding of some animal proteins with digoxin tag.

Probes have been synthesized (in particular, probe **XXVII** (Scheme 9) [152]) with the hexose residue (D-Man or D-Glc) bound through 4-OH with the Tfd-label by the 2-aminopropyl residue. The latter was bound with biotin through a number of different length linkers built with alternating tartrate, oligoethylene glycol, and/or succinate. Upon the detection by chemiluminescence, the labeled protein could be isolated by cleavage of the tartrate residue with periodate. An integral membrane protein, glucose transporter, exemplified that for probing of carbohydrate proteins on the intact cell surface using the streptavidin detecting system the linker had to contain no less than 60-70 atoms. Before the detecting, the sought protein was isolated from the mixture of cell proteins by immunoprecipitation [152].

The probe for detecting galectins, a combination with chemically selective binding and visualization, presents a new approach for PAL of lectins [153]. Galectins belong to the family of lectins that specifically bind with the structures containing monovalent β-galactosides [149]. Probe **XXVIII (**Scheme 9) is a lactose analog; 3′-OH is chosen for introduction of the Bp-label considering the crystalline structure of galectins 1 and 3; the diethylene glycol spacer compensates for the hydrophobicity of the photophore. The method is based on the copper-catalyzed selective 1,3-cyclic binding of azides to alkins. The reaction is "bioorthogonal", i.e., it does not occur with the groups specific for biosystems. After the irradiation (360 nm, 30 min), the probe mixture with galectin is treated by the rhodaminyl-containing alkin **XXIX** (Scheme 9). On the electrophoregram, the visualization is realized by scanning of laser-excited fluorescence. The selective PAL of galectins 1 and 3 in the mixture with cytochrome *c* and glyceraldehyde-3-phosphate dehydrogenase has been demonstrated [153].

Examples of photoaffinity probing for studies on various biological systems have been presented. In the late 1990s and early 2000s, the number of reports in this field was slightly decreased, but for the recent three-four years the number of works increased. Obviously, PAL combined with new powerful analytical methods and computer modeling remains a most important and promising approach in structural biology.

The author is grateful to Dr. J. G. Molotkovsky for his valuable comments and recommendations on reading the manuscript.

REFERENCES

1. Knowles, J. K. (1972) *Acc. Chem. Res.*, **5**, 150-160.
2. Bayley, H. (1983) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., and Burdon, R. H., eds.) Vol. 12, Elsevier, Amsterdam-New York-Oxford.
3. Brunner, J. (1989) *Meth. Enzymol.*, **172**, 628-687.
4. Brunner, J. (1993) *Ann. Rev. Biochem.*, **62**, 483-514.
5. Kotzyba-Hibert, F., Kapfer, I., and Goeldner, M. (1995) *Angew. Chem., Int. Ed.*, **34**, 1296-1312.
6. Fleming, S. A. (1995) *Tetrahedron*, **51**, 12479-12520.
7. Hatanaka, Y., Nakayama, H., and Kanaoka, Y. (1996) *Rev. Heteroatom Chem.*, **14**, 213-243.
8. Dorman, G., and Prestwich, G. D. (1994) *Biochemistry*, **33**, 5661-5673.
9. Knoll, F., Kolter, T., and Sandhoff, K. (2000) *Meth. Enzymol.*, **311**, 568-600.
10. Dorman, G., and Prestwich, G. D. (2000) *Trends Biotechnol.*, **18**, 64-77.
11. Hatanaka, Y., and Sadakane, Y. (2002) *Curr. Top. Med. Chem.*, **2**, 271-288.
12. Singh, A., Thornton, E. R., and Westheimer, F. H. (1962) *J. Biol. Chem.*, **237**, PC3006-PC3008.
13. Jahn, O., Eckart, K., Tezval, H., and Spiess, J. (2004) *Analyt. Bioanalyt. Chem.*, **378**, 1031-1036.
14. Shacham, S., Marantz, Y., Bar-Haim, S., Kalid, O., Warshaviak, D., Avisar, N., Inbal, B., Heifetz, A., Fichman, M., Topf, M., Naor, Z., Noiman, S., and Becker, O. M. (2004) *Proteins*, **57**, 51-86.
15. Zhang, H., and Karlin, A. (1997) *Biochemistry*, **36**, 15856-15864.
16. Foucaud, B., Perret, P., Grutter, T., and Goeldner, M. (2001) *Trends Pharmacol. Sci.*, **22**, 170-173.
17. Fleet, G. W. J., Porter, R. R., and Knowles, J. R. (1969) *Nature*, **224**, 511-512.
18. Smith, R. A. G., and Knowles, J. R. (1973) *J. Am. Chem. Soc.*, **95**, 5072-5073.
19. Galardy, R. E., Craig, L. C., and Printz, M. P. (1973) *Nature New Biol.*, **242**, 127-128.
20. Chowdhry, V., Vaughan, R., and Westheimer, F. H. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1406-1408.

21. Stoffel, W., Schtreiber, C., and Scheefers, H. (1978) *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 923-931.
22. Brunner, J., Senn, H., and Richards, F. M. (1980) *J. Biol. Chem.*, **255**, 3313-3318.
23. Goeldner, M., and Hirth, C. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6439-6442.
24. Nielsen, P. E., Hansen, J. B., Thomsen, T., and Burhardt, O. (1983) *Experientia*, **39**, 1063-1072.
25. Young, M. J. T., and Platz, M. S. (1989) *Tetrahedron Lett.*, **30**, 2199-2202.
26. Goeldner, M., Hawkinson, J. E., and Casida, J. E. (1989) *Tetrahedron Lett.*, **30**, 823-826.
27. Kolpashchikov, D. M., Zakharenko, A. L., Dezhurov, S. V., Rechkunova, N. I., Khodyreva, S. N., Degtyarev, S. Kh., Litvak, V. V., and Lavrik, O. I. (1999) *Bioorg. Khim.*, **25**, 129-136.
28. Turro, N. J. (1980) *Ann. N. Y. Acad. Sci.*, **346**, 1-17.
29. Gritsan, N. P., Yuzawa, T., and Platz, M. S. (1997) *J. Am. Chem. Soc.*, **119**, 5059-5060.
30. Lwowski, W. (1980) *Ann. N. Y. Acad. Sci.*, **346**, 491-500.
31. Borden, W. T., Gritsan, N. P., Hadad, C. M., Karney, W. L., Kemnitz, C. R., and Platz, M. S. (2000) *Acc. Chem. Res.*, **33**, 765-771.
32. Cai, S. X., Glenn, D. J., and Keana, J. F. W. **(**1992) *J. Org. Chem.*, **57**, 1299-1304.
33. Platz, M., Admasu, A. S., Kwiatkowski, S., Crocker, P. J., Imai, N., and Watt, D. S. (1991) *Bioconjugate Chem.*, **2**, 337-341.
34. Jahn, O., Hofmann, B. A., Brauns, O., Spiess, J., and Eckart, K. (2002) *Int. J. Mass Spectrom.*, **214**, 37-51.
35. Hino, N., Okazaki, Y., Kobayashi, T., Hayashi, A., Sakamoto, K., and Yokoyama, S. (2005) *Nature Meth.*, **2**, 201-206.
36. Farrelli, I. S., Toroney, R., Hazen, J. L., Mehl, R. A., and Chin, J. W. (2005) *Nature Meth.*, **2**, 377-384.
37. Kage, R., Leeman, S. E., Krause, J. E., Costello, C. E., and Boyd, N. D. (1996) *J. Biol. Chem.*, **271**, 25797-25800.
38. Bisello, A., Adams, A. E., Mierke, D. F., Pellegrini, M., Rosenblatt, M., Suva, L. J., and Chorev, M. (1998) *J. Biol. Chem.*, **273**, 22498-22505.
39. Behar, V., Bisello, A., Rosenblatt, M., and Chorev, M. (1999) *Endocrinology*, **140**, 4251-4261.
40. Behar, V., Bisello, A., Bitan, G., Rosenblatt, M., and Chorev, M. (2000) *J. Biol. Chem.*, **275**, 9-17.
41. Macdonald, D., Mierke, D. F., Li, H., Pellegrini, M., Sachais, B., Krause, J. E., Leeman, S. E., and Boyd, N. D. (2001) *Biochemistry*, **40**, 2530-2539.
42. Rihakova, L., Deraet, M., Auger-Messier, M., Perodin, J., Boucard, A. A., Guillemette, G., Leduc, R., Lavigne, P., and Escher, E. (2002) *J. Recept. Signal Transduction Res.*, **22**, 297-313.
43. Clement, M., Martin, S. S., Beaulieu, M. E., Chamberland, C., Lavigne, P., Leduc, R., Guillemette, G., and Escher, E. (2005) *J. Biol. Chem.*, **280**, 27121-27129.
44. Weber, P. J. A., and Becksickinger, A. G. (1997) *J. Peptide Res.*, **49**, 375-383.
45. Tate, J. J., Persinger, J., and Bartholomew, B. (1998) *Nucleic Acids Res.*, **26**, 1421-1426.
46. Gilbert, B. A., and Rando, R. R. (1995) *J. Am. Chem. Soc.*, **117**, 8061-8066.
47. Hatanaka, Y., Hashimoto, M., and Kanaoka, Y. (1998) *J. Am. Chem. Soc.*, **120**, 453-454.
48. Perodin, J., Deraet, M., Auger-Messier, M., Boucard, A. A., Rihakova, L., Beaulieu, M.-E., Lavigne, P., Parent, J.-L., Guillemette, G., Leduc, R., and Escher, E. (2002) *Biochemistry*, **41**, 14348-14356.
49. Ji, Z., Hadac, E. M., Henne, R. M., Patel, S. A., Lybrand, T. P., and Miller, L. J. **(**1997) *J. Biol. Chem.*, **272**, 24393-24401.
50. Jahn, O., Tezval, H., Spiess, J., and Eckart, K. (2003) *Int. J. Mass Spectrom.*, **228**, 527-540.
51. Li, C., Xu, W., Vadivel, S. K., Fan, P., and Makriyannis, A. (2005) *J. Med. Chem.*, **48**, 6423-6429.
52. Palnitkar, S. S., Bin, B., Jimenez, L. S., Morimoto, H., Williams, P. G., Paul-Pletzer, K., and Parness, J. (1999) *J. Med. Chem.*, **42**, 1872-1880.
53. Paul-Pletzer, K., Yamamoto, T., Ikemoto, N., Jimenez, L. S., Morimoto, H., Williams, P. G., Ma, J., and Parness, J. (2005) *Biochem. J.*, **387**, 905-909.
54. Kramer, W., Girbig, F., Corsiero, D., Burger, K., Fahrenholz, F., Jung, C., and Muller, G. (2003) *Biochim. Biophys. Acta*, **1633**, 13-26.
55. Kramer, W., Corsiero, D., Girbig, F., and Jahne, G. (2006) *Biochim. Biophys. Acta – Biomembranes*, **1758**, 45-54.
56. Kramer, W., Sauber, K., Baringhaus, K.-H., Kurz, M., Stengelin, S., Lange, G., Corsiero, D., Girbig, F., Konig, W., and Weyland, C. (2001) *J. Biol. Chem.*, **276**, 7291-7301.
57. Lee, H. K., Zheng, Y. F., Xiao, X.-Y., Bai, M., Sakakibara, J., Ono, T., and Prestwich, G. D. (2004) *Biochem. Biophys. Res. Commun.*, **315**, 1-9.
58. Ohgami, N., Ko, C. D., Thomas, M., Scott, P. M., Chang, C. C. Y., and Chang, T.-Y. **(**2004) *Proc. Natl. Acad. Sci. USA*, **101**, 12473-12478.
59. Wu, Z., Hasan, A., Liu, T., Teller, D. C., and Crabb, J. W. (2004) *J. Biol. Chem.*, **279**, 27357-27364.
60. Borhan, B., Souto, M. L., Imai, H., Shichida, Y., and Nakanishi, K. (2000) *Science*, **288**, 2209-2212.
61. Demers, A., Mcnicoll, N., Febbraio, M., Servant, M., Marleau, S., Silverstein, R., and Ong, H. (2004) *Biochem. J.*, **382**, 417-424.
62. Alqwai, O., Poelarends, G., Konings, W. N., and Georges, E. (2003) *Biochem. Biophys. Res. Commun.*, **311**, 696-701.
63. Pleban, K., Kopp, S., Csaszar, E., Peer, M., Hrebicek, T., Rizzi, A., Ecker, G. F., and Chiba, P. (2005) *Mol. Pharmacol.*, **67**, 365-374.
64. Vaughan, R. A., Parnas, M. L., Gaffaney, J. D., Lowe, M. J., Wirtz, S., Pham, A., Reed, B., Dutta, S. M., Murray, K. K., and Justice, B. J. (2005) *J. Neurosci. Meth.*, **143**, 33-40.
65. Vaughan, R. A., Gaffaney, J. D., Lever, J. R., Reith, M. E. A., and Dutta, A. K. (2001) *Mol. Pharmacol.*, **59**, 1157-1164.
66. Dohlman, H. G., Caron, M. G., Strader, C. D., Amlaiky, N., and Lefkowitz, R. J. (1988) *Biochemistry*, **27**, 1813-1817.
67. Janovick, J. A., Haviv, F., Fitzpatrick, T. D., and Conn, P. M. (1993) *Endocrinology*, **133**, 942-945.
68. Kennedy, A. P., Mangum, K. C., Linden, J., and Wells, J. N. (1996) *Mol. Pharmacol.*, **50**, 789-798.
69. Li, Y. M., Marnerakis, M., Stimson, E. R., and Maggio, J. E. (1995) *J. Biol. Chem.*, **270**, 1213-1220.
70. Boucard, A. A., Sauve, S. S., Guillemette, G., Escher, E., and Leduc, R. (2003) *Biochem. J.*, **370**, 829-838.
71. Hein, L., Barsh, G. S., Pratt, R. E., Dzau, V. J., and Kobilka, B. K. (1995) *Nature*, **377**, 744-747.

72. Nikiforovich, G. V., and Marshall, G. R. (2001) *Biochem. Biophys. Res. Commun.*, **286**, 1204-1211.
73. Boucard, A. A., Wilkes, B. C., Laporte, S. A., Escher, E., Guillemette, G., and Leduc, R. (2000) *Biochemistry*, **39**, 9662-9670.
74. Johannesson, P., Lindeberg, G., Johansson, A., Nikiforovich, G. V., Gogoll, A., Synnergren, B., Le Greves, M., Nyberg, F., Karlen, A., and Hallberg, A. **(**2002) *J. Med. Chem.*, **45**, 1767-1777.
75. Yamano, Y., Ohyama, K., Kikyo, M., Sano, T., Nakagomi, Y., Inoue, Y., Nakamura, N., Morishima, I., Guo, K.-F., Hamakubo, T., and Inagami, T. (1995) *J. Biol. Chem.*, **270**, 14024-14030.
76. Deraet, M., Rihakova, L., Boucard, A., Perodin, J., Sauve, S., Mathieu, A. P., Guillemette, G., Leduc, R., Lavigne, P., and Escher, E. (2002) *Can. J. Physiol. Pharmacol.*, **80**, 418-425.
77. Fillion, D., Deraet, M., Holleran, B. J., and Escher, E. (2006) *J. Med. Chem.*, **49**, 2200-2209.
78. Behar, V., Bisello, A., Rosenblatt, M., and Chorev, M. (1999) *Endocrinology*, **140**, 4251-4261.
79. Behar, V., Bisello, A., Bitan, G., Rosenblatt, M., and Chorev, M. (2000) *J. Biol. Chem.*, **275**, 9-17.
80. Dong, M., Pinon, D. I., Cox, R. F., and Miller, L. J. (2004) *J. Biol. Chem.*, **279**, 1167-1175.
81. Dong, M., Pinon, D. I., Cox, R. F., and Miller, L. J. (2004) *J. Biol. Chem.*, **279**, 31177-31182.
82. Tan, Y. V., Couvineau, A., van Rampelbergh, J., and Laburthe, M. (2003) *J. Biol. Chem.*, **278**, 36531-36536.
83. Tan, Y. V., Couvineau, A., and Laburthe, M. (2004) *J. Biol. Chem.*, **279**, 38889-38894.
84. Pham, V. I., and Sexton, P. M. (2004) *J. Pept. Sci.*, **10**, 179-203.
85. Assil, I. Q., Qi, L. J., Arai, M., Shomali, M., and Abou-Samra, A. B. (2001) *Biochemistry*, **40**, 1187-1195.
86. Klose, J., Fechner, K., Beyermann, M., Krause, E., Wendt, N., Bienert, M., Rudolph, R., and Rothemund, S. (2005) *Biochemistry*, **44**, 1614-1623.
87. Kraetke, O., Holeran, B., Berger, H., Escher, E., Bienert, M., and Beyermann, M. (2005) *Biochemistry*, **44**, 15569-15577.
88. Unwin, N. (2005) *J. Mol. Biol.*, **346**, 967-989.
89. Grutter, T., and Changeux, J. P. (2001) *Trends Biochem. Sci.*, **26**, 459-463.
90. Mourot, A., Grutter, T., Goeldner, M., and Kotzyba-Hibert, F. (2006) *ChemBioChem*, **7**, 570-583.
91. Brejc, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van der Oost, J., Smit, A. B., and Sixma, T. K. (2001) *Nature*, **411**, 269-276.
92. Galzi, J. L., Revah, F., Black, D., Goeldner, M., Hirth, C., and Changeux, J. P. **(**1990) *J. Biol. Chem.*, **265**, 10430-10437.
93. Grutter, T., Ehret-Sabatier, L., Kotzyba-Hibert, F., and Goeldner, M. (2000) *Biochemistry*, **39**, 3034-3043.
94. Middleton, R. E., and Cohen, J. B. **(**1991) *Biochemistry*, **30**, 6987-6997.
95. Kotzyba-Hibert, F., Grutter, T., and Goeldner, M. (1999) *Mol. Neurobiol.*, **20**, 45-59.
96. Addona, G. H., Kloczewiak, M. A., and Miller, K. W. (1999) *Analyt. Biochem.*, **267**, 135-140.
97. Leite, J. F., Blanton, M. P., Shahgholi, M., Dougherty, D. A., and Lester, H. A. **(**2003) *Proc. Natl. Acad. Sci. USA*, **100**, 13054-13059.
98. Galzi, J., Revah, F., Bouet, F., Menez, A., Goeldner, M., Hirth, C., and Changeux, J. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5051-5055.
99. Grutter, T., Bertrand, S., Kotzyba-Hibert, F., Bertrand, D., and Goeldner, M. (2002) *ChemBioChem*, **3**, 652-658.
100. Mourot, A., Rodrigo, J., Kotzyba-Hibert, F., Bertrand, S., Bertrand, D., and Goeldner, M. (2006) *Mol. Pharmacol.*, **69**, 452-461.
101. Mordvintsev, D. Yu., Polyak, Ya. L., Levtsova, O. V., Tourleigh, Ye. V., Kasheverov, I. E., Shaitan, K. V., Utkin, Yu. N., and Tsetlin, V. I. (2005) *Comput. Biol. Chem.*, **29**, 398-411.
102. Utkin, Y. N., Hatanaka, Y., Franke, P., Machold, J., Hucho, F., and Tsetlin, V. I. (1995) *J. Protein Chem.*, **4**, 197-203.
103. Ziebell, M. R., Nirthanan, S., Husain, S. S., Miller, K. W., and Cohen, J. B. **(**2004) *J. Biol. Chem.*, **279**, 17640-17649.
104. Gupta, C. M., Radhakrishnan, R., and Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 4315-4319.
105. Wiegandt, H. (1985) in *Gangliosides. New Comprehensive Biochemistry* (Neuberger, A., and van Deenen, L. L. M., eds.) Vol. 10, Elsevier, Amsterdam, pp. 199-260.
106. Hakomori, S.-I. (1990) *J. Biol. Chem.*, **265**, 18713-18716.
107. Yates, A. J., and Rampersaud, A. (1998) *Ann. N. Y. Acad. Sci.*, **845**, 57-71.
108. Vanmeer, G., and Lisman, Q. (2002) *J. Biol. Chem.*, **277**, 25855-25858.
109. Bektas, M., and Spiegel, S. (2003) *Glycoconjugate J.*, **20**, 39-47.
110. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.*, **67**, 199-225.
111. Helms, J. B., and Zurzolo, C. (2004) *Traffic*, **5**, 247-254.
112. Fra, M. A., Masserini, M., Palestini, P., Sonnino, S., and Simons, K. (1995) *FEBS Lett.*, **375**, 11-14.
113. Mauri, L., Prioni, S., Loberto, N., Chigorno, V., Prinetti, A., and Sonnino, S. (2004) *Glycoconjugate J.*, **20**, 11-23.
114. Sonnino, S., Chirgorno, V., Acquotti, D., Pitto, M., Kirchner, G., and Tettamanti, G. (1989) *Biochemistry*, **28**, 77-84.
115. Prinetti, A., Marano, N., Prioni, S., Chigorno, V., Mauri, L., Casellato, R., Tettamanti, G., and Sonnino, S. (2000) *Glycoconjugate J.*, **17**, 223-232.
116. Loberto, N., Prioni, S., Prinetti, A., Ottico, E., Chigorno, V., Karagogeos, D., and Sonnino, S. (2003) *J. Neurochem.*, **85**, 224-233.
117. Prioni, S., Mauri, L., Loberto, N., Casellato, R., Chigorno, V., Karagogeos, D., Prinetti, A., and Sonnino, S. (2004) *Glycoconjugate J.*, **21**, 461-470.
118. Palestini, P., Pitto, M., Tedeschi, G., Ferraretto, A., Parenti, M., Brunner, J., and Masserini, M. (2000) *J. Biol. Chem.*, **275**, 9978-9985.
119. Wendeler, M., Hoernschemeyer, J., Hoffmann, D., Kolter, T., Schwarzmann, G., and Sandhoff, K. (2004) *Eur. J. Biochem.*, **271**, 614-627.
120. Shapiro, R. E., Specht, C. D., Collins, B. E., Woods, A. S., Cotter, R. J., and Schpaar, R. L. (1997) *J. Biol. Chem.*, **272**, 30380-30386.
121. Weber, T., and Brunner, J. (1995) *J. Am. Chem. Soc.*, **117**, 3084-3095.
122. Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R., and Brunner, J. (1995) *J. Biol. Chem.*, **270**, 17575-17581.

123. Durrer, P., Galli, C., Hoenke, S., Corti, C., Gluck, R., Vorherr, T., and Brunner, J. (1996) *J. Biol. Chem.*, **271**, 13417-13421.
124. Janssen, M. J. F. W., Voorst, F., Ploeger, G. E. J., Larsen, P. M., Larsen, M. R., Kroon, A. I. P. M., and Kruijff, B. (2002) *Biochemistry*, **41**, 5702-5711.
125. Vodovozova, E. L., Tsibizova, E. V., and Molotkovsky, J. G. (2001) *J. Chem. Soc., Perkin Trans. 1*, No. 18, 2221-2228.
126. Zaitseva, L. G., Ovchinnikova, T. V., Vodovozova, E. L., Molotkovsky, J. G., Polyakov, N. B., Titov, M. I., Esipov, S. E., and Grinkevich, V. A. (2002) *Bioorg. Khim.*, **28**, 411-425.
127. Delfino, J. M., Schtreiber, S. L., and Richards, M. F. (1993) *J. Am. Chem. Soc.*, **115**, 3458-3474.
128. Ogawa, Y., Hahn, W., Garnier, P., Higashi, N., Massotte, D., Metz-Boutigue, M.-H., Rousseau, B., Sunamoto, J., Ourisson, G., and Nakatani, Y. (2001) *Angew. Chem. Int. Ed.*, **40**, 944-946.
129. Zegers, M. M. P., Kok, J. W., and Hoekstra, D. (1997) *Biochem. J.*, **328**, 489-498.
130. Chaudhary, A., Chen, J., Gu, Q.-M., Witke, W., Kwiatkowski, D. J., and Prestwich, G. D. (1998) *Chem. Biol.*, **5**, 273-281.
131. Tall, E., Dorman, G., Garcia, P., Runnels, L., Shah, S., Chen, J., Profit, A., Gu, Q.-M., Chaudhary, A., Prestwich, G. D., and Rebecchi, M. J. (1997) *Biochemistry*, **36**, 7239-7248.
132. Giraldo, A. M. V., Castello, P. R., Flechaa, F. L. G., Moeller, J. V., Delfino, J. M., and Rossi, J. P. F. C. (2006) *FEBS Lett.*, **580**, 607-612.
133. Persinger, J., and Bartholomew, B. (1996) *J. Biol. Chem.*, **271**, 33039-33046.
134. Knorre, D. G., Kudryashova, N. V., and Lavrik, O. I. (1998) *Uspekhi Khim.*, **67**, 486-502.
135. Zakharenko, A. L., Khodyreva, S. N., Rechkunova, N. I., Safronov, I. V., Pyshnyi, D. V., Degtyarev, S. Kh., and Lavrik, O. I. (1998) *Biochemistry (Moscow)*, **63**, 929-934.
136. Lavrik, O. I., Nasheuer, H. P., Weisshart, K., Wold, M. S., Prasad, R., Beard, W. A., Wilson, S. H., and Favre, A. (1998) *Nucleic Acids Res.*, **26**, 602-607.
137. Kolpashchikov, D. V., Ivanova, T. M., Boghachev, V. S., Nasheuer, H. P., Weisshart, K., Favre, A., Pestryakov, P. E., and Lavrik, O. I. (2000) *Bioconjug. Chem.*, **11**, 445-451.
138. Lavrik, O. I., Kolpashchikov, D. V., Weisshart, K., Nasheuer, H. P., Khodyreva, S. N., and Favre, A. (1999) *Nucleic Acids Res.*, **27**, 4235-4240.
139. Kolpashchikov, D. V., Khodyreva, S. N., Khlimankov, D. Y., Wold, M. S., Favre, A., and Lavrik, O. I. (2001) *Nucleic Acids Res.*, **29**, 373-379.
140. Lebedeva, N. A., Rechkunova, N. I., Dezhurov, S. V., Degtyarev, S. Kh., and Lavrik, O. I. (2004) *Bioorg. Khim.*, **30**, 369-374.
141. Lavrik, O. I., Prasad, R., Sobol, R. W., Horton, J. K., Ackerman, E. J., and Wilson, S. H. (2001) *J. Biol. Chem.*, **276**, 25541-25548.
142. Dezhurov, S. V., Khodyreva, S. N., Plekhanova, E. S., and Lavrik, O. I. (2005) *Bioconjug. Chem.*, **16**, 215-222.
143. Dyrkheeva, N. S., Khodyreva, S. N., Sukhanova, M. V., Safronov, I. V., Dezhurov, S. V., and Lavrik, O. I. (2006) *Biochemistry (Moscow)*, **71**, 200-210.
144. Mal'tseva, E. A., Rechkunova, N. I., Petruseva, I. O., Sil'nikov, V. N., Vermeulen, V., and Lavrik, O. I. (2006) *Biochemistry (Moscow)*, **71**, 270-278.
145. Sverdlov, E. D., and Tsarev, S. A. (1980) *Bioorg. Khim.*, **6**, 1110-1113.
146. Godovikova, T. S., Berezovskii, M. V., and Knorre, D. G. (1995) *Bioorg. Khim.*, **21**, 858-867.
147. Godovikova, T. S., Knorre, V. D., Maksakova, G. A., and Sil'nikov, V. N. **(**1996) *Bioconj. Chem.*, **7**, 343-348.
148. Knorre, D. G., Kudryashova, N. V., Popova, T. V., Shakirov, M. M., Mal'shakova, V. S., Shpenev, O. E., Savinkova, L. K., Serebryakova, M. V., and Godovikova, T. S. (2005) *Bioorg. Khim.*, **31**, 372-384.
149. Gabius, H.-J., Siebert, H.-C., Andre, S., Jimenez-Barbero, J., and Rudiger, H. (2004) *ChemBioChem.*, **5**, 740-764.
150. Lauc, G., Flogel, M., Diehl-Seifert, B., Schroder, H. C., and Muller, W. E. G. (1994) *Glycoconj. J.*, **11**, 541-549.
151. Lauc, G., Lee, R. T., Dumic, J., and Lee, Y. C. (2000) *Glycobiology*, **10**, 357-364.
152. Hashimoto, M., Yang, J., and Holman, G. D. (2001) *ChemBioChem.*, **2**, 52-59.
153. Ballell, L., Alink, K. J., Slijper, M., Versluis, C., Liskamp, R. M. J., and Pieters, R. J. (2005) *ChemBioChem.*, **6**, 291-295.