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Benzophenone Photophores in Biochemistry†

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ABSTRACT: The photoactivatable aryl ketone derivatives have been rediscovered as biochemical probes in the last 5 years. The expanding use of benzophenone (BP) photoprobes can be attributed to three distinct chemical and biochemical advantages. First, BPs are chemically more stable than diazo esters, aryl azides, and diazirines. Second, BPs can be manipulated in ambient light and can be activated at 350–360 nm, avoiding protein-damaging wavelengths. Third, BPs react preferentially with unreactive C–H bonds, even in the presence of solvent water and bulk nucleophiles. These three properties combine to produce highly efficient covalent modifications of macromolecules, frequently with remarkable site specificity. This Perspective includes a brief review of BP photochemistry and a selection of specific applications of these photoprobes, which address questions in protein, nucleic acid, and lipid biochemistry.

Benzophenone (BP[1]) photochemistry has attracted the attention of biochemists for over 2 decades. The first application of the photogenerated triplet state of BPs in biological systems was reported in 1974 (Galardy et al., 1974). Several years later, the BP photophore was employed as a tethered, photoactivatable reagent to functionalize specific remote C–H bonds in steroids and to map conformations of flexible chains in solution, micelles, and membranes (Breslow, 1980, 1986). In 1983, only scattered examples of the successful use of BP-containing photoaffinity probes were mentioned (Bayley, 1983). Three years later, evidence began to accumulate showing numerous advantages of these photolabeling derivatives for obtaining covalent modifications of nucleotide binding sites (Williams et al., 1986). The surge of citations in the biochemical literature since 1986 indicates that the utility of photoactivatable aryl ketone derivatives has been rediscovered (Dormán & Prestwich, 1993). In particular, the development of the photolabile amino acid analog, *p*-benzoyl-L-phenylalanine (Kauer et al., 1986), led to a plethora of new studies of peptide–protein interactions. In several cases, the previously employed aryl azide or diazirine probes (Bayley, 1983) have been supplanted by the corresponding BP-containing probe, thereby increasing labeling efficiency in hydrophobic binding sites.

The expanding use of BP photoprobes can be attributed to three distinct chemical and biochemical advantages. First, BPs are chemically more stable than diazo esters, aryl azides, and diazirines. Second, BPs can be manipulated in ambient light and can be activated at 350–360 nm, avoiding protein-damaging wavelengths. Third, BPs react preferentially with unreactive C–H bonds, even in the presence of solvent water and bulk nucleophiles. These three properties combine to produce highly efficient covalent modifications of macromolecules, frequently with remarkable site specificity. These

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[1] Abbreviations: ATPγBP, *N*-[(4-benzoylphenyl)methyl]phosphoramide analog of ATP; BP, benzophenone; L-Bpa, 4-benzoyl-L-phenylalanine; BP-m^{7}GTP, [γ-^{32}P]-[[(4-benzoylphenyl)methyl]amino]-7-methylguanosine 5′-triphosphate; BPIA, 4-(2-iodoacetamido)benzophenone; Bz$_2$εADP, 3′(2′)-*O*-(4-benzoylbenzoyl)-1,N^6-ethenoadenosine 5′-diphosphate; BzAF, 1-[(4-benzoylbenzoyl)amino]fluorescein; BzATP, 3′(2′)-*O*-(4-benzoylbenzoyl)-ATP; BzCDP–choline, 3′(2′)-*O*-(4-benzoylbenzoyl)-[^{32}P]CDP–choline; BzCTP, 3′-*O*-(4-benzoylbenzoyl)cytidine 5′-phosphate; [^{3}H]BZDC-IP$_3$, [[([^{3}H]-*p*-benzoyldihydrocinnamyl)amino]propyl]-IP$_3$; [^{3}H]BZDC-NHS, succinimido [2,3-^{3}H$_2$]-*p*-benzoyldihydrocinnamate; CaM, calmodulin; CCK, cholecystokinin; IP$_3$, inositol 1,4,5-triphosphate; IP$_4$, inositol 1,3,4,5-tetraphosphate; MBP, 4-maleimidobenzophenone; MLCK, myosin light-chain kinase; 8-N$_3$ATPγBP, 8-azidoadenosine triphosphate BP derivative; SE, squalene epoxidase.

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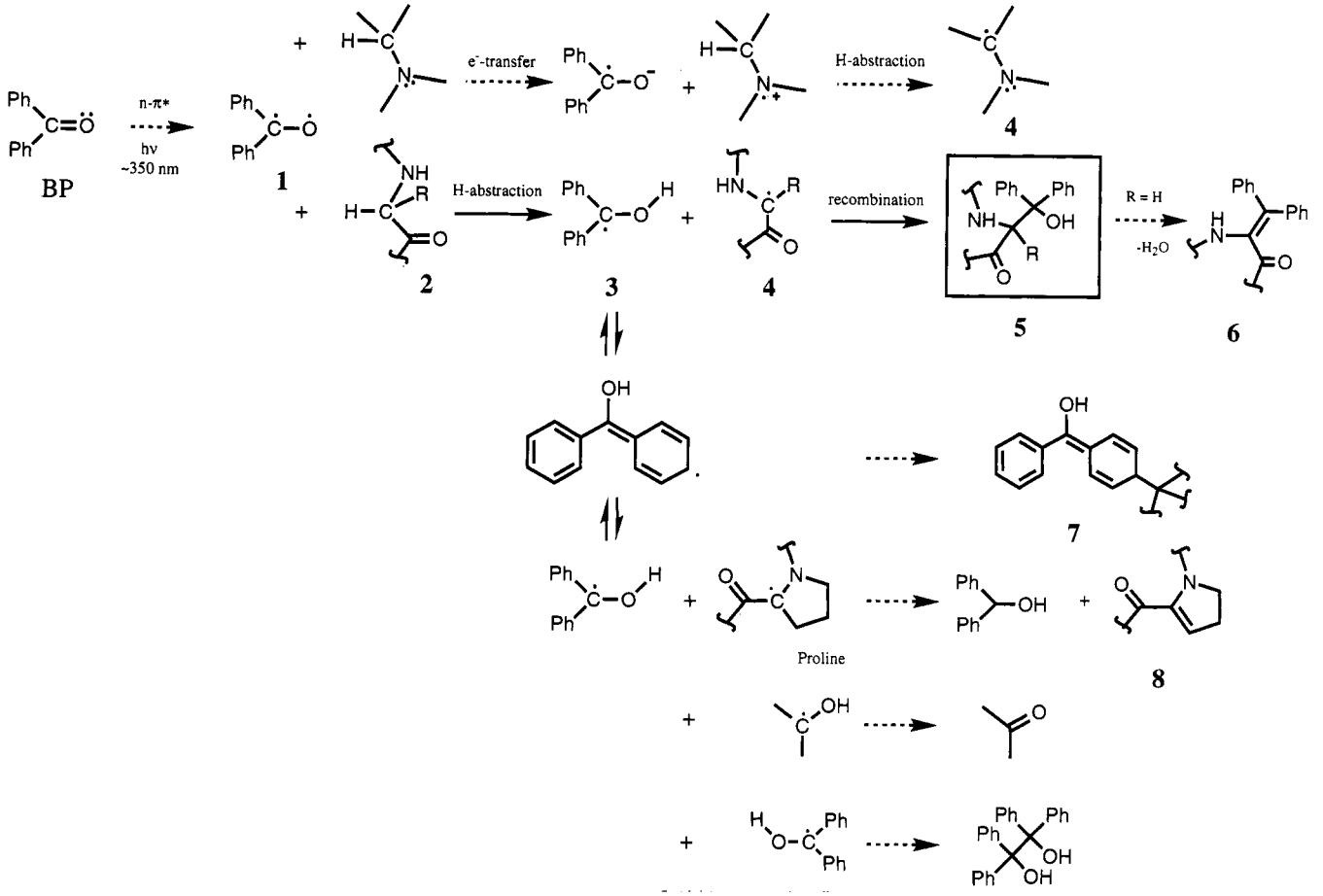
Scheme 1: Photochemistry of BP Chromophore and Radical Recombination Pathways



advantages generally outweigh the disadvantages of additional bulkiness and hydrophobicity resulting from the introduction of the BP group. In this Perspective, we provide a brief review of the photochemistry of the BP photophore, followed by an overview of specific applications of BP photoprobes in protein, nucleic acid, and lipid biochemistry.

OVERVIEW OF BP PHOTOCHEMISTRY

Carbonyl photochemistry and concomitant hydrogen-atom-transfer reactions are widely used in modern synthetic chemistry (Turro, 1978). Focusing on the BP photophore, absorption of a photon at ~350 nm results in the promotion of one electron from a nonbonding sp^2-like n-orbital on oxygen to an antibonding π^*-orbital of the carbonyl group (Scheme 1). In the diradicaloid triplet state (**1**), the electron-deficient oxygen n-orbital is electrophilic and therefore interacts with weak C–H σ-bonds (**2**), resulting in hydrogen (H) abstraction to complete the half-filled n-orbital. When amines or similar heteroatoms are proximal to the excited carbonyl, an electron-transfer step may occur, followed by proton abstraction from an adjacent alkyl group and a radical 1,2-shift. The ketyl (**3**) and alkyl (**4**) radicals that are formed readily recombine to generate a new C–C bond, producing benzpinacol-type compounds (**5**). This basic process, which is also used for remote site functionalization, is based on the observation that the diradicaloid species can only attack geometrically accessible C–H bonds. The reactant and substrate must spend sufficient time at the interactive distance, thus furnishing the primary source of the site specificity. The selectivity is then increased by the stereoelectronic factors of the frontier molecular orbitals favoring certain angles and planes for the attack (Scheme 2).

Lifetime. The lifetime of the excited state containing two unpaired electrons is much longer than that of the singlet state. Thus, the triplet state may last 80–120 μs in the absence of an abstractable H, but it may be 100 times shorter in the presence of a suitably oriented C–H bond. Increasing concentrations of an H-donor decrease the lifetime proportionally according to the Stern–Volmer equation,

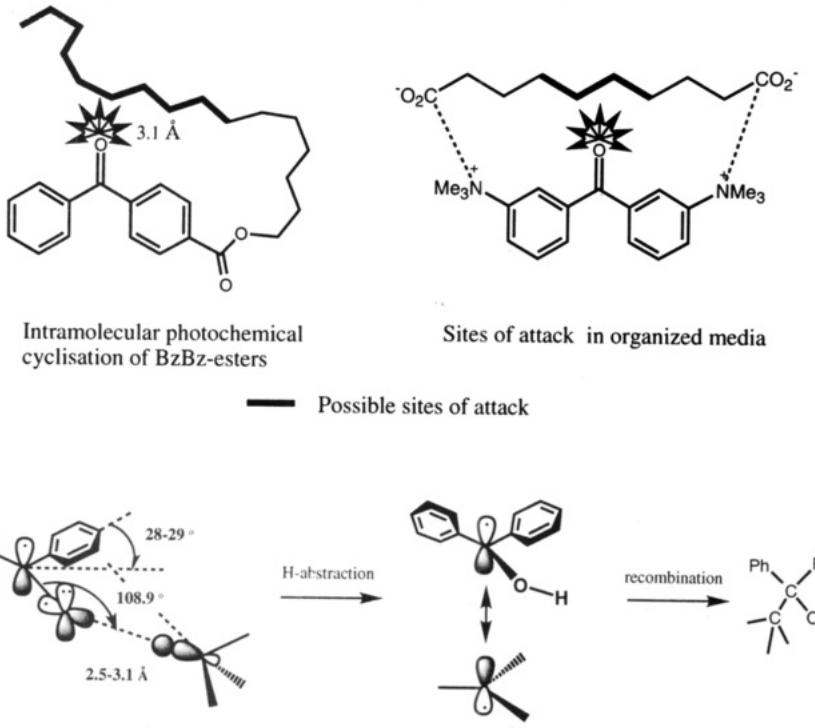
$$\frac{1}{\tau} = \frac{1}{\tau_0} + k_2[\mathrm{RH}]$$

where τ_0 is the excited-state lifetime in the absence of RH (H-donor), τ is the excited-state lifetime in the presence of RH (H-donor), and k_2 is the second-order rate coefficient.

The triplet state readily relaxes to the ground state if it does not find an H-donor with the required geometry. This relaxation process does not require a scavenger, and this constitutes one of the major advantages of this photophore. Other photoreactive groups are activated in a photodissociative mode, that is, activation is irreversible. In contrast, BP probes may relax electronically, maintaining their binding and photoactivatable properties and then undergoing many excitation–relaxation cycles until a favorable geometry for covalent modification is achieved. The probability of achieving an optimal geometry for covalent attachment can be further improved by using a flexible linker, although more rigid linkers should give superior data in the topographic mapping of an active site. For low-affinity binding, the prolonged lifetime and irradiation time may cause excessive nonspecific labeling. However, for high-affinity interactions (micromolar and below), labeling selectivity can be anticipated to be quite high.

The lifetime of the recombination is considerably shorter. The radicals formed by H-abstraction remain in close

Scheme 2: Geometric Control of Hydrogen Abstraction: Attacking Sites and Regioelectronic Control



proximity in the transition state, and only minimal motion is required to fulfill the stereoelectronic requirements for bond formation.

Flexibility and Proximity as Factors in Photoprobe Design. Intramolecular BP photochemistry with long flexible chains provides multiple sites for attack (Winnik, 1981). On the basis of modeling and experimental data, the reactive volume of the BP moiety was approximated as a sphere with a radius of 3.1 Å centered on the ketone oxygen (Scheme 2), and the overall process is controlled by rotational accessibility. Intermolecular reactions are very slow, are completely diffusion-controlled, and lack regioselection. However, in model reactions that employ hydrogen bonds or ion pairs as organizing forces, selective attack was observed. For example, when symmetrical BP-3,3′-dicarboxylic acid or a related positively charged BP derivative (Scheme 2) was photolyzed in the presence of a long-chain dicarboxylic acid (Breslow, 1980), the H-abstraction occurred from the internal methylenes of the diacid substrate. The incorporation of two binding points into the long-chain substrate restricted its motion relative to the photochemical BP probe, just as ligands show reduced conformational mobility when bound to an enzyme or a receptor active site. In the excited state, the BP ring system is required to be nearly planar, imparting significant rigidity. This rigid system can be extended with rigid linkers to reposition the photophore and thereby alter the efficiency and site of the resulting attachment. For biochemical studies, the section of linker size and orientation is crucial, since the primary goal is to label the binding site for the ligand while minimizing nonspecific modifications. Typically, the BP photophore in biochemical probes would be coupled to active ligands via linkers bearing either moderately flexible (1–3 CH_2 units, O, NH) or moderately rigid (C=O, COO) functionalities. In conclusion, BP photochemistry in biochemical systems is most regioselective when the flexibility is limited to only that which is necessary to achieve efficient H-abstraction.

Regioelectronic Control: The Role of Fine-Tuning. The factors controlling BP photoreactivity impart macroscopic geometric control; the geometry of the attack imparts the microscopic geometric control, with preferred angles of attack providing the fine-tuning. Regiochemical and stereoelectronic control results from the preferred molecular orbital geometry of the photochemically excited triplet. The conformation of the n–π^* triplet state is close to planarity; the half-filled n-orbital lies in this ideal plane, while the π^*-orbital is perpendicular to it (Scheme 2).

Ab initio calculations determined an idealized transition-state model, in which the C_{CO}–O–H angle of the biradical attack was found to be 108.9°, the O–H–C_{CH} bonds were almost collinear, and the attack occurred in-plane (Severance et al., 1987). Although the observed values deviate slightly from that model (Wagner et al., 1994), they still represent a significant geometric control. Radical recombination may then serve to impart a second stereoelectronic control, since the recombination requires the p-orbitals to be collinear for maximum overlap. Finally, the radicals can approach from either face, giving either a racemic or diastereomeric mixture in a chiral environment.

Reactivity and Substituent Effects. Apart from the geometric factors, the reactivity and the efficiency of covalent attachment strongly depend on the chemical and electronic environment of the photophore and the H-donor moiety (Turro, 1978). The strength of the bond being broken in the H-donor and the relative stabilities of the resulting alkyl radicals are the major determining factors. In general, the homolytic cleavage of C–H bonds, induced by the triplet biradical, is favored over the O–H bonds. In the case of N–H bonds, the electrophilic excited state abstracts an electron first, and this event is followed by the H-abstraction from the adjacent C–H group, as described earlier (Scheme 1).

In biological systems, the most effective H-donors include backbone C–H bonds in amino acids, polypeptides, and carbohydrates. Methylene groups of lipids and amino acid side chains also provide abstractable hydrogens. In the absence of any orientational preference, the reactivity order for C–H bonds is >NCH_x> -SCH_x> methine > C=CCH_2> -CH_2- > -CH_3. Particularly reactive sites include the electron-

rich tertiary centers such as C_γ–*H* of leucine and C_β–*H* of valine and those CH_2 groups adjacent to heteroatoms in Lys, Arg, and Met (O'Neil et al., 1989). Insertion into vinylic or aromatic C–H bonds has not been reported.

In biochemical probes, BP either is connected to an active ligand or is used to replace a portion of the ligand, partially mimicking its geometry. The substituents on BP can affect the photochemistry significantly. Thus, electron-withdrawing groups increase the efficiency of H-abstraction. Electron-donating groups and electron delocalization into aromatic and conjugated systems cause a partial shift of the electron transition from n–π^* to π–π^*, and the latter triplet state becomes much less reactive toward H-abstraction. The overall quantum yield (Φ) varies from 0.05 to 0.4, with the higher values for the simple n–π^* transition. Finally, substituents can affect the steric accessibility; the placement of alkyl groups in *ortho* or *meta* positions of the BP increases the probability of intramolecular interactions that reduce the effectiveness of the probe.

Recombination Products and Identification of Modification Sites. Analysis of the site of photocovalent attachment provides information about the location of the binding domain, and therefore a stable cross-linked complex is desired. Frequently, modification leads to altered proteolytic or chemical fragmentation patterns. The absence of a modified amino acid phenylthiohydantoin derivative during Edman degradation may also signal where modification has occurred, and in optimal cases, the amino acid that contains the benzhydrol derivative can be detected. The radical recombination product is suppressed only if a ring is the target, due to the additional steric hindrance (Scheme 1). Thus, when proline was found as a main cross-linking site, a dehydroproline moiety (**8**) arose from a second H-transfer to the ketyl radical, resulting in an elimination. This process reduced the radioactivity incorporation (Cole & Yount, 1990). Abstraction from secondary alcohols can lead to a second ketyl radical that may form a ketone by the removal of a second hydrogen. After recombination, the adduct is generally stable. However, if a Gly residue has been modified, the resulting benzhydrol can undergo elimination (dehydration), giving a highly conjugated species (**6**) (Scheme 1).

In general, benzpinacol formation accompanies the heteroradical recombination as a radical dimerization, albeit in low yield. With laser flash photolysis, the cross-linking can occur on the 2- or 4-position of the phenyl ring, forming highly colored species, referred to as light-absorbing transients (**7**) (Demeter & Bérces, 1989).

BIOCHEMICAL APPLICATIONS OF TETHERED BENZOPHENONES

Biomimetic Chemistry in Organized Media. An early bridge between organic chemistry and biochemistry was created through the use of membrane mimics as semiorganized media for organic reactions (Breslow, 1980, 1986). The geometric control of this biomimetic process occasionally resulted in site-directed cross-linking between the flexible chains, which were organized into micelles. Indeed, the photoinduced covalent modification at a defined depth in the hydrophobic membrane has emerged as a very powerful tool for studying the structure of membranes with or without guests, such as proteins or small molecules. Traditional mapping of hydrophobic domains with carbenes and nitrenes, including a discussion of the main features of designing probes for mapping the membrane–protein interface, has been summarized previously (Brunner, 1989).

In general, the BP photophore is linked to alkyl chains with polar terminal functionalities to create an amphiphilic probe. Thus, BP-containing long-chain fatty acids in micelles were irradiated with polyunsaturated fatty acids, and the H-abstraction occurred predominantly from the CH_2 bonds in allylic positions (Markovic et al., 1990). Amphiphilic choline esters of 4-alkylbenzophenonecarboxylic acids (**9**, Chart 1) also allowed the mapping of fatty acid–detergent chain–chain interactions (Gogoll & Schäfer, 1987). A more accurate mapping of the micelle depth was carried out using phosphatidylcholine analogs, in which BP replaced a portion in the center of the myristoyl side chain (Lala & Kumar, 1993). These derivatives (**12**, Chart 1) photoattached primarily to the C-8 and C-9 positions of the neighboring myristoyl chain.

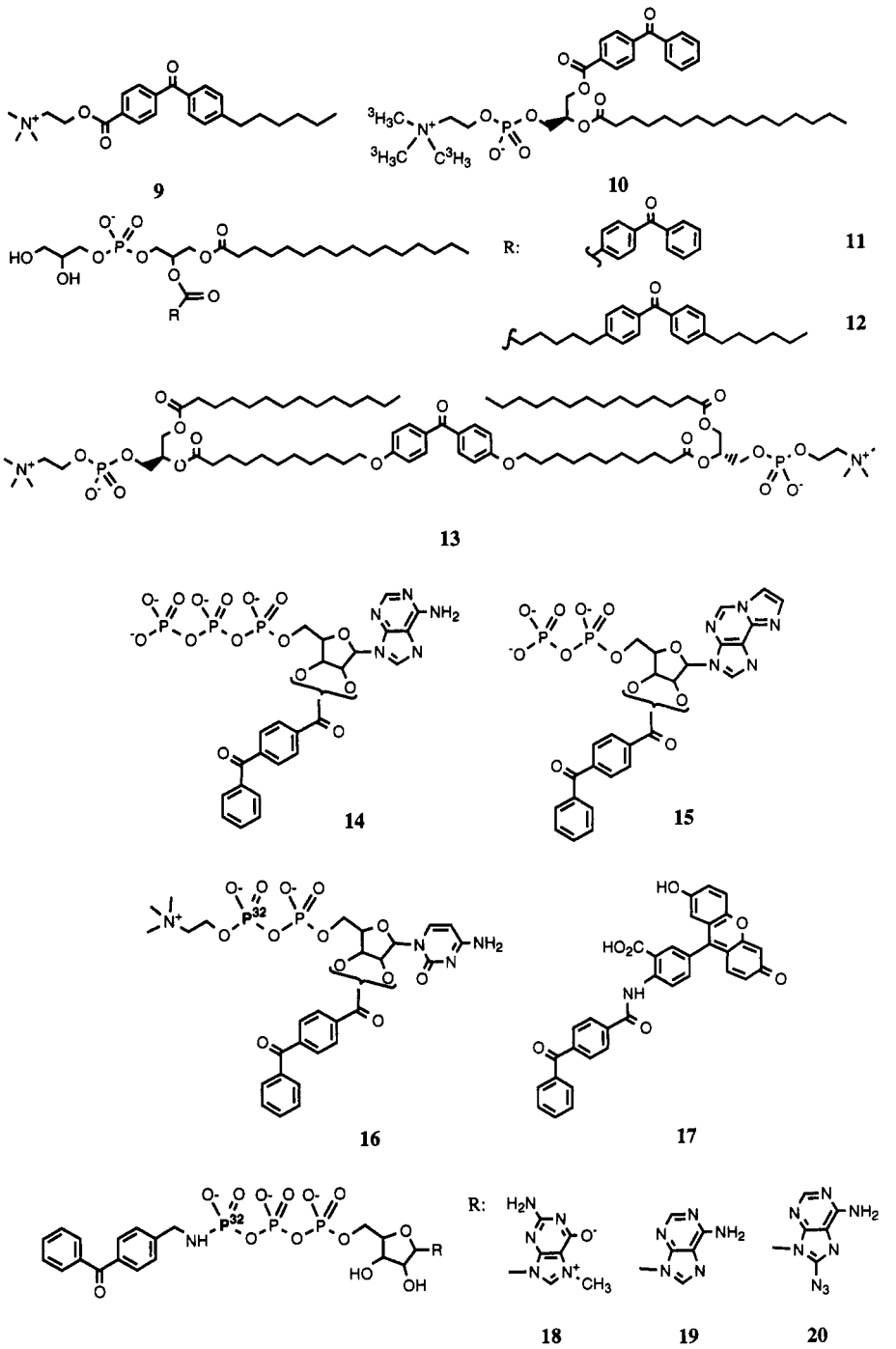
These phospholipid-type probes appeared to offer much promise for mapping the hydrophobic transmembrane regions of proteins. The phosphatidylcholine derivative, 2-[(4-benzoylphenyl)acetoxy]-1-palmitoylphosphatidylcholine (**11**, Chart 1), effectively labeled the lactose permease protein from the cytoplasmic membrane of *Escherichia coli* (Page & Rosenbusch, 1986). This probe exhibited membrane-domain-specific labeling, unlike the corresponding aryl azido derivative. A similar probe, 1-palmitoyl-2-(*p*-benzoylbenzoyl)phosphocholine (**10**, Chart 1) (Montecucco & Schiavo, 1986), was employed for labeling the membrane-associated region of Na^+- and K^+-activated ATPase and Ca^{2+}-activated ATPase, albeit with modest efficiency. Ourisson and co-workers reported a bilayer mimic lipid probe that fixed the photoactivation site in the center of the hydrophobic region (Yamamoto et al., 1993). As shown in Chart 1, two 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine units were connected to a bifunctional BP photophore via a bisether linkage (**13**), and the probe was placed in a bilayer. This bilayer-spanning probe labeled the hydrophobic region of guest molecules, such as cholesterol, showing very efficient and regiospecific attachment within a range of 3 Å of the middle of the bilayer.

Mapping of ATP and GTP Binding Sites. The early applications of 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP), still one of the most widely used BP probes, were reviewed through 1986 (Williams et al., 1986). Many new applications of this probe (**14**, Chart 1) include its use as an active-site-directed, photoactivated irreversible inhibitor, as well as a photoaffinity label. This probe labels the protein at a distance of 6–7 Å from the ribose 3′(2′)-oxygen. For example, single-site photoinactivation was induced in three different ATP-hydrolyzing F_1-subunits of ATPases (F_1-ATPase) (Aloise et al., 1991). In each case, photocovalent binding was also observed on additional binding domains in the presence of Mg^{2+}.

Photolabeling of the isolated α- and β-subunits and the $\alpha_3\beta_3$ core complex of F_1-ATPase from bacterial plasma membrane showed that covalent binding of BzATP to either α- or β-subunits prevented assembly to the hexamer complex (Bar-Zvi et al., 1992). The preassembled $\alpha_3\beta_3$ core also collapsed upon irradiation in the presence of the photoprobe. In contrast, the native enzyme, which contains other minor subunits (γ, δ, and ϵ), remained intact upon covalent modification, suggesting that these additional components serve to stabilize the complex.

A different cross-linking strategy was developed to study these interactions in chloroplast F_1 (Musier & Hammes, 1987). Intramolecular covalent bonds were created between the γ-subunit and the $\alpha_3\beta_3$ core, restricting free motion. Two heterobifunctional alkylamides bearing a maleimide functionality at one end and a BP photophore at the N-terminal

Chart 1: Lipid Bilayer and Nucleotide Photoprobes



end were first attached in the dark to the sulfhydryl-rich γ-subunit. Next, irradiation established cross-links with the large $\alpha_3\beta_3$ core.

The Coleman group more recently introduced a fluorescent nucleotide site-specific photoaffinity label, 1-[(4-benzoylbenzoyl)amino]fluorescein (BzAF, **17**, Chart 1), a potent competitive inhibitor of the ATPases that saturably binds to both sites of adenylate kinase (Pal et al., 1990). After the complexed site was saturated with MgATP, BzAF photolabeled the uncomplexed site with 85% incorporation of radioactivity. Covalent modification of both sites with BzAF led to a ternary complex, and conformational changes during complex formation were revealed by the fluorescence (Pal et al., 1992).

In another application, BzATP induced an increase in plasma membrane permeability in mouse fibroblast 3T6 cells at a lower concentration than ATP itself, as a consequence of binding to the plasma P_2 purinoceptors. This activity was maintained after photolysis, demonstrating that the photoincorporation occurred at the binding site (Erb et al., 1990). A similar study was performed on P_{2Y} purinergic receptors isolated from turkey erythrocyte membrane (Boyer et al., 1990).

The ATP binding domain in ryanodine receptors was also mapped using BzATP (Zarka & Shoshan-Barmatz, 1993). Upon photoactivation, BzATP irreversibly binds to the ATP regulatory binding site and reduces the ryanodine concentration required for Ca^{2+} release by 1000-fold.

A pyrimidine nucleotide of this type, 3′-*O*-(4-benzoylbenzoyl)cytidine 5′-phosphate (BzCTP, not shown), was synthesized enzymatically from BP-carboxylic acid and labeled CTP (Abeijon et al., 1986). Bacterial CMP-*N*-acetyl-

neuraminic acid synthetase accepted this substrate mimic, and the ^{32}P-labeled photoprobe covalently modified the enzyme. This experiment suggested that pyrimidine-requiring active sites were also candidates for BP photoaffinity mapping.

In another family of nucleotide probes, the BP was coupled to the terminal phosphate via a tethered amide linkage. Chart 1 shows the structure of [γ-^{32}P]-[[(4-benzoylphenyl)methyl]-amino]-7-methylguanosine 5′-triphosphate (**18**), an analog of the cap structural unit of the eukaryotic messenger RNAs at the 5′-position (Thalmann & Blaas, 1991). This cap analog served as a probe for studying the initiation of protein synthesis. Using this simple nucleotide probe, cap binding proteins were labeled specifically both from whole cells and from different nuclei implicated in this signaling process. The same probe was used to map the cap binding site of initiation factor eIF-4E from rabbit reticulocyte postribosomal supernatant and human erythrocyte lysate (Chavan et al., 1990).

A similarly functionalized adenosine moiety, the *N*-[(4-benzoylphenyl)methyl]phosphoramide analog of ATP (ATPγBP, **19**, Chart 1), was used for labeling the Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) activase binding site from tobacco (Salvucci et al., 1993). Two distinct regions of this enzyme were covalently modified by the photoprobe. One site was adjacent to the characteristic P-loop, which was expected to be part of the binding region of γ-phosphate. A second modified peptide showed high amino acid sequence identity to adenylate kinase.

This ATP probe (**19**) was further modified (Rajagopalan et al., 1993) to give an 8-azidoadenosine derivative (8-N_3-ATPγBP, **20**, Chart 1) and the analogous GTP derivative (8-N_3GTPγBP). These bidentate reagents created a new class of photo-cross-linkers, combining two mechanistically different photoreactive groups, e.g., the aryl azido and BP photophores. Sequential photochemical activation of the noncovalent complexes gave cross-linking patterns different from those found when the monodentate parent probes were used. A complex of the GTP bifunctional probe with the tubulin receptor was briefly irradiated at 254 nm to activate the aryl azide, resulting in covalent modification of the β-subunit. Prolonged photolysis at 360 nm led to incorporation of the BP into the α-subunit.

Two photoactivatable fluorescent etheno-ADP analogs, 3′-(2′)-*O*-(4-benzoylbenzoyl)-1,N^6-ethenoadenosine 5′-diphosphate ($Bz_2\epsilon$ADP, **15**, Chart 1) and its 2-deoxy analog (not shown), were prepared to study the actin–myosin interaction (Cremo & Yount, 1987). On the basis of earlier results for myosin subfragment 1 (SF_1), photoreactive ligands were first anchored in the nucleotide binding site with bifunctional thiol-selective reagents; irradiation then resulted in photocovalent attachment. The photochemical cross-linking efficiency (40–50%) was detected by the alteration of the fluorescent emission wavelength in the covalent complex possessing a reduced benzhydrol unit. The $Bz_2\epsilon$ADP-labeled, fluorescent SF_1 fragment bound specifically to F-actin, allowing further studies of the resulting ternary complex.

The same 50-kDa SF_1 heavy chain was labeled and sequenced by using BzATP (Mahmood et al., 1989). The BP carbonyl group inserted into a C–H bond from Ser-324. The corresponding 50-kDa labeled fragments were isolated from different conformations (6*S*, extended, and 10*S*, folded) of unphosphorylated chicken gizzard myosin. Before irradiation, the affinity probe BzATP or BzADP was first trapped as a Co(II) orthovanadate complex to prevent nonspecific labeling (Cole & Yount, 1990). The ketyl radical labeled Pro-324 at a position similar to that shown for rabbit skeletal myosin, with reduced incorporation of radioactivity resulting from cleavage of the benzhydrol.

Photoaffinity labeling of choline phosphotransferase from rat liver microsomes was carried out with either [^{32}P]CDP–choline or its BP analog, 3′(2′)-*O*-(4-benzoylbenzoyl)-[^{32}P]-CDP–choline (BzCDP–choline, **16**, Chart 1) (Ishidate et al., 1992). The incorporation of radioactivity was dependent on Mg^{2+} (or Mn^{2+}) and was inhibited by the presence of Ca^{2+}.

Inositol Polyphosphate Receptor Affinity Probes. Inositol 1,4,5-triphosphate (IP_3) is an intracellular messenger that acts to release calcium from intracellular stores. A photoaffinity analog of IP_3, [[([^3H]-(*p*-benzoyldihydrocinnamyl)-amino]propyl]-IP_3 ([^3H]BZDC-IP_3, **21**, Chart 2), was synthesized to map the IP_3 receptor (IP_3R) (Mourey et al., 1993). An aminopropyl tether was employed to append the photolabile moiety to the P-1 phosphate, thus maintaining binding affinity and specificity for the IP_3R, which requires the presence of the 4,5-diphosphate moiety. [^3H]BZDC-IP_3 showed high affinity for the rat brain IP_3R and efficiently and selectively labeled the IP_3 binding site, previously localized to the N-terminal 1–788 extracellular loop by mutagenesis experiments.

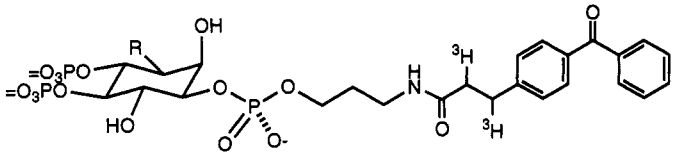
[^3H]BZDC-IP_3 was first prepared by reductive tritiation of the conjugated double bond in [[(*p*-benzoylcinnamyl)-amino]propyl]-IP_3, giving a specific activity of 3–4 Ci/mmol. A higher specific activity probe was required for the detection of low-abundance receptors. Thus, the synthetic strategy was modified by preparing a radiolabeled heterobifunctional photolabile reagent, succinimido [2,3-$^3H_2$]-*p*-benzoyldihydrocinnamate ([^3H]BZDC-NHS) (**39**, Chart 3; J. D. Olszewski, J. F. Marecek, D. A. Ahern, G. Dormán, and G. D. Prestwich, unpublished results). This reagent was then coupled to a variety of aminopropyl-tethered inositol polyphosphate probes. This methodology was first successfully employed to prepare [^3H]BZDC-IP_3 with high specific activity for a study of IP_3R in insect neural tissues, rat and catfish olfactory cilia, and rat liver nuclei.

Inositol 1,3,4,5-tetraphosphate (IP_4, **22**, Chart 2) may regulate Ca^{2+} reentry into the cell and may modulate the IP_3-sensitive pools. The optically active P-1-aminopropyl-tethered IP_4 analog (Estevez & Prestwich, 1991) was similarly linked to the BZDC photophore. The [^3H]BZDC-IP_4 photoprobe specifically labeled rat brain IP_4 and IP_6 receptors (A. A. Profit, A. B. Theibert, and G. D. Prestwich, unpublished results), which had been previously purified and selectively photolabeled with a radioiodinated azidosalicylamide derivative of tethered IP_4 (Theibert et al., 1992).

Transfer RNA Photoprobes. BP derivatives have been employed to photolabel oligonucleotides in a study of the topography of the peptidyl transferase regions in ribosomes (Steiner et al., 1988). A photoreactive 3-(4-benzoylphenyl)-propionyl-Phe-tRNA was synthesized and incorporated into 23S RNA, a key element in the peptide transferase activity of the ribosome. Highly efficient (>70%) photocovalent cross-linking was found at the P-site at low Mg^{2+} concentrations, while the A-site was preferred for high amounts of Mg^{2+}. Many antibiotics (e.g., chloramphenicol and tetracycline) inhibit peptide bond formation in ribosomes; thus, abrogation of the photolabeling following antibiotic preincubation confirmed that labeling was proximal to the peptidyl transferase site (Kuechler et al., 1989).

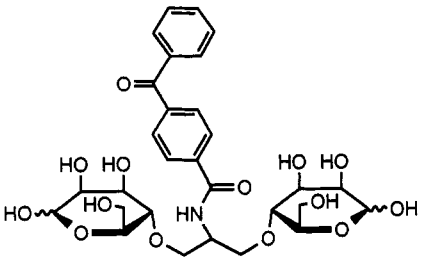
Carbohydrate Probes. BP-derivatized carbohydrates were suggested as useful probes for multiple purposes in studying gelling mechanisms, glycoproteins, membrane transport, and polysaccharide mobility (Hall & Yalpani, 1980). To date,

Chart 2: Photoprobes for IP_3R, Mannose Transporter, Diuretic Receptor, Progestin Binding Proteins, and Squalene Epoxidase



R: -OH $InsP_3$ **21**

$-OPO_3^{2-}$ $InsP_4$ **22**



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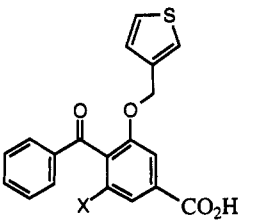
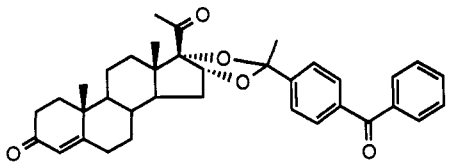


X: SO_2NH_2

24



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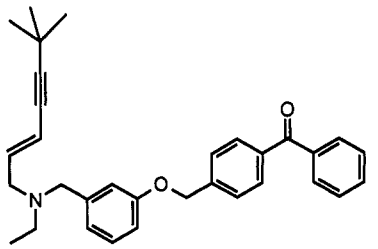

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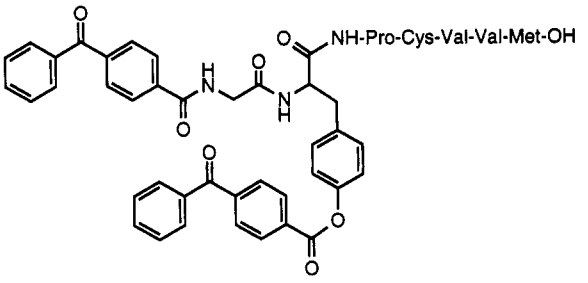


27

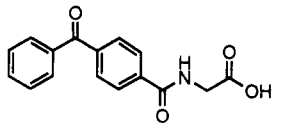
however, the only literature precedent is a BP-modified 1,3-bis(D-mannos-4-yloxy)-2-propylamine (**23**, Chart 2) (Holman et al., 1988). This probe has been tested as an exofacial photoaffinity label for sugar-transport systems. In erythrocyte membranes, the covalent labeling was efficient and specific (displaceable by D-glucose). In a rat adipocyte model, insulin treatment increased the intensity of observed photolabeling 7-fold, resulting from a higher availability of transporter proteins. After photolysis in the presence of insulin, 30% of the labeled transporters was internalized into the light microsomes of the cell.

Receptors for Diuretics in Kidney Membranes. In Henle's loop of the mammalian kidney, Na^+, Cl^-, and water reabsorption can be inhibited by diuretics. Salt transport through the apical membrane of the epithelial cells is the possible target of these drugs. A BP analog of the diuretic bumetanide, [^{3}H]-4-benzoyl-5-sulfamoyl-3-[(3-thienylmethyl)oxy]benzoic acid (**24**, Chart 2), selectively labeled a 150-kDa protein from dog kidney cortex. Efficient labeling requires Na^+, K^+, and Cl^- ions, suggesting that this protein was part of the cotransport system. A mouse kidney membrane-labeling pattern revealed that the corresponding 150-kDa protein is a dimer (Haas et al., 1991).

Progesterone Receptor Photoprobes. The progestin 16α, 17α-dioxolane ketal of BP (**25**, Chart 2) was synthesized as a novel type of molecular probe to map the progesterone receptor (Kym et al., 1993). This BP analog showed high affinity in bioassays and induced 29% inactivation upon photolysis with a rat uterine preparation.

Squalene Epoxidase Photoaffinity Labels. A BP-containing photoaffinity analog of NB-598 (**26**, Chart 2), a potent inhibitor of vertebrate squalene epoxidase (SE), has been

Chart 3: BP-Modified Amino Acids and Heterobifunctional Reagents



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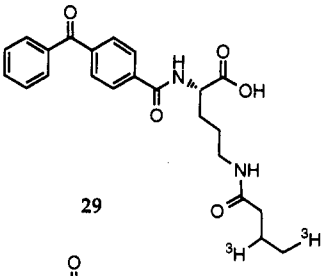

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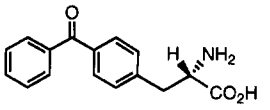

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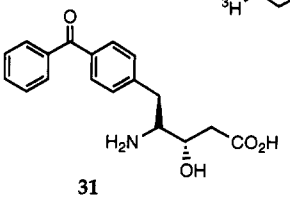


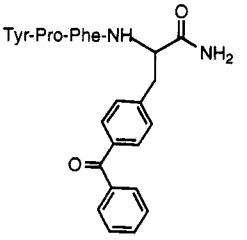
31

Leu-Arg-Arg-Ala-**Bpa**-Leu-Gly

Bpa-Arg-Arg-Ala-Ser-Leu-Gly

Leu-Arg-Arg-**Bpa**-Ser-Leu-Gly

32a-c



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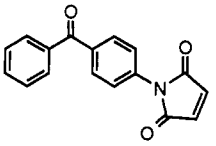
Thiol-reactive cross-linkers:



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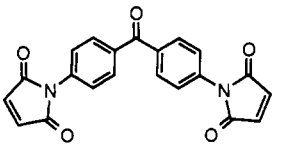

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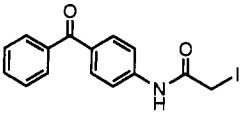

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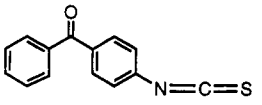
Amine-reactive cross-linkers:



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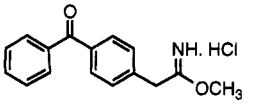

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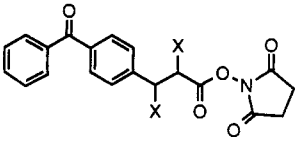

39

X= ^{1}H, ^{3}H

prepared (M. Ceruso and G. D. Prestwich, unpublished results). The BP can act as an isosteric replacement for either the bisthienyl or the allylamine moiety, and these biaryl substitutions have been effective in the design of inhibitors of fungal and vertebrate SE (Nussbaumer et al., 1993; Abe et al., 1994). Preliminary results suggest that this new photolabel will be valuable in defining the inhibitor binding site(s) of rat and pig liver SE.

Catalytic Site of Farnesyl Protein Transferase. Farnesyl protein transferase, which has α and β heterodimer subunits (total = 95 kDa), binds both the protein and farnesyl diphosphate. Protein recognition occurs via a C-terminal *CAAX* amino acid sequence. Coleman and co-workers attached one or two benzophenones directly to the *CAAX* motif (**27**, Chart 2). These analogs were very good substrates. The mono-BP analog specifically labeled the β-subunit, while the di-BP-*CAAX* labeled the whole dimer (Ying et al., 1994).

Cholecystokinin Receptors. Cholecystokinin (CCK), a 33 amino acid peptide in the gastrointestinal tract, binds to a peripheral type of receptor (CCK_A) in the pancreas, while in the central nervous system it has affinity for a central receptor (CCK_B) (Thiele & Fahrenholz, 1993). The most active fragment of CCK is a sulfated octapeptide (CCK-8s), which was modified to give a photoprobe containing a BP photophore at the N^{α}-position and a radiolabel at the N^{δ}-position of an ornithine tether (**29**, Chart 3). This probe selectively labeled a 56-kDa glucoprotein within the CCK_B receptor from rat cerebellar cortex and a 80–100-kDa peptide in membranes from rat pancreas (CCK_A receptor). This receptor was also labeled by an N-terminal-modified BP analog of the 1–10 amino acid portion of CCK (Klueppelberg et al., 1990).

Additional BP Derivatives of Polypeptides. Modification of short peptides using 4-azido and 4-benzoyl derivatives of benzoic acid NHS ester was examined, and superior results

for photo-cross-linking were obtained with the BP-containing probes. In one example, the N-terminal octapeptide of pilin was N-terminally derivatized with the BP-containing reagent and then photo-cross-linked in 50–60% yield to bovine serum albumin, which was in contrast to the 10% incorporation for the aryl azide derivative (Parker & Hodges, 1985).

The development of the tritium-labeled *p*-benzoyldihydrocinnamyl succinimido ([^{3}H]BZDC-NHS) ester reagent (J. D. Olszewski, D. A. Ahern, G. Dormán, and G. D. Prestwich, unpublished results, **39**, Chart 3) has facilitated access to a variety of photoaffinity analogs of polypeptides. This reagent allows preferential derivatization of the ϵ-amino groups of Lys residues over N-terminal amino groups, when used in methanolic DMF solution with a tertiary amine base. Moreover, the use of this reagent facilitates active-site mapping of the target receptor for a given polypeptide, since the photophore itself bears a high specific activity (30–50 Ci/mmol), a long half-life, and a safely handled tritium label. This reagent is now available as a commercial product.

Four peptides under study in our laboratories illustrate the utility of this new reagent. First, we have prepared [^{3}H]-BZDC-GGR, a photolabeled tripeptide that shows activation of an egg-pumping behavior in mud crabs at femtomolar concentrations (G. Dormán, D. Rittschof, and G. D. Prestwich, unpublished results). Second, the unique Lys-27 of the 33-residue *Heliothis zea* pheromone biosynthesis activating neuropeptide (PBAN) has been derivatized, and the resulting BZDC-PBAN has been shown to be equipotent to the underivatized peptide hormone in eliciting pheromone biosynthesis in isolated abdomens of two female moths (J. Elliott, R. Jurenka, W. L. Roelofs, and G. D. Prestwich, unpublished results). Third, an [^{3}H]BZDC-Lys-labeled analog of the human thrombin receptor activating peptide, SFLLRNP-NDKY, was recently prepared and found to be active in platelet aggregation assays, and it is in use for identification of the peptide–thrombin receptor binding domain (J. Elliott, B. S. Coller, K. T. Springer, and G. D. Prestwich, unpublished data). Finally, partially selective modification of one of the three Lys residues of *Manduca sexta* diuretic hormone with [^{3}H]-BZDC-NHS gave a biologically active photoaffinity label, [^{3}H]BZDC-Mas-DH, which is currently in use for mapping the peptide binding domain of recombinant Mas-DHR (G. Du, J. Reagan, and G. D. Prestwich, unpublished results).

SITE-DIRECTED PHOTOLABELING WITH POLYPEPTIDES CONTAINING 4-BENZOYLPHENYLALANINE AND RELATED AMINO ACIDS

Heterobifunctional cross-linking reagents such as the [^{3}H]-BZDC-NHS reagent are very useful for preparing photolabile peptides. However, this technique is limited by the necessity to attach the photophore at chemically reactive groups within the polypeptide. Photoactivatable amino acids (Eberle & de Graan, 1985) can circumvent this problem, since solid-phase synthetic techniques allow the replacement of any amino acid in a polypeptide with a photolabile analog. In early work, (benzoylbenzoyl)glycine (**28**, Chart 3) was prepared and incorporated into an octapeptide for photo-cross-linking to albumin (Parker & Hodges, 1985). However, the majority of the effort in this area now focuses on the use of 4-benzoyl-L-phenylalanine (L-Bpa, **30**, Chart 3) as a photoactivatable replacement for aromatic amino acid residues in a sequence of interest.

Calmodulin (CaM) and Protein–Protein Interactions. Synthesis and resolution of L-Bpa and its incorporation into several different positions in a synthetic 17-residue peptide known to bind to calmodulin (CaM) by solid-phase peptide synthesis inaugurated the work in this area (Kauer et al., 1986). Several model peptides were examined to reveal the mechanism by which CaM activates and modulates many different peptides, such as protein kinases or adenylate cyclases (O'Neil & DeGrado, 1989). In a 17-residue model peptide that bound to bovine testes CaM in a Ca^{2+}-dependent manner, Trp-3 was changed to the isosteric Bpa. In another model peptide, Leu-13 was substituted by Bpa-13. Modification of a smooth muscle myosin light-chain kinase (MLCK) fragment at the 3-position gave MLCK-Bpa. The photolabeling of CaM was specific and very efficient in each case (70–90%). On both sides of the central α-helical cavity of CaM, certain methionines were labeled. Met-144 was exclusively labeled with Bpa-3 and MLCK-Bpa, while Bpa-13 was attached to Met-71 and Met-144 in a 10:1 ratio. A doubly modified (Bpa-3,13) 17-mer was also assembled, and this bivalent probe labeled Met-71 on one side of the cavity followed by Met-124 on the other side of the cavity. The Met–71-Met-124 distance was shorter than anticipated, indicating that the domains, which are connected by the α-helical tether, moved closer upon ligand binding. This flexibility allows the CaM to recognize and interact with a wide variety of proteins.

Protease Inhibitor Photolabels. A Bpa statine analog (**31**, Chart 3) was prepared for the active-site mapping of a protease (Kuzmic et al., 1990). The unnatural D-Bpa and a BP analog of threonine were also synthesized and assembled into pentapeptides mimicking pepstatin and cyclosporin fragments. A D-Bpa-containing pentapeptide (Boc-Val-D-Bpa-Sta-Ala-Sta-OMe) inhibited porcine pepsin in 75% yield upon irradiation at 350 nm.

Protein Kinase Substrates. A heptamer peptide (kemptide), a known substrate for the cAMP-dependent protein kinase, was modified by replacing a Ser, a Leu, and an Ala with racemic DL-Bpa, respectively (**32a–c**, Chart 3); the diastereomeric peptides were separated by HPLC (Miller, 1991). Photoaffinity labeling with **32a** was specific and efficient (90% incorporation of radioactivity) and caused complete inactivation of the kinase. Photocovalent attachment sites on the kinase were identified as Gly-125 and Met-127.

Selective Opioid Receptor Label. A photoreactive tetrapeptide analog (4-BMA, **33**, Chart 3) of morphiceptin possessed L-Bpa amide in place of the C-terminal prolinamide. The affinity of the μ-opioid receptor for this peptide was found to be comparable to that for the parent compound, but significantly lower than those for the δ- and κ-receptors (Herblin et al., 1987) Thus, the covalent peptide–receptor complex was completely inactivated only toward DAGO, a μ-receptor-specific synthetic pentamer.

Substance P Receptor Mapping. A Bpa-modified undecapeptide fragment of substance P ([^{125}I]Bpa^8-SP = RPK-PQQP*X*GLMNH$_2$, *X* = Bpa) was synthesized in which the Phe-8 residue in the C-terminal end was replaced by L-Bpa (Boyd et al., 1991). Irradiation of [^{125}I]Bpa^8-SP with the membrane-bound SP receptors from a rat submaxillary gland led to 70% incorporation of the label into two polypeptides (46 and 53 kDa). Enzymatic studies revealed that the lower molecular mass protein was a proteolysis product, since both peptides contained identical N-linked carbohydrate units; labeling of both fragments was similarly inhibited by displacement with nonhydrolyzable GTP analogs (Kage et al., 1993).

Insulin Receptor Mapping. A purified human insulin receptor was mapped with a Bpa-containing insulin analog

(BBpa–insulin), which was biotinylated to enable high-sensitivity detection (Shoelson et al., 1993). Since Phe-B25 was crucial in receptor recognition, and since *para* substituents of this amino acid were well-tolerated, Bpa was inserted at this position in a C-terminal octapeptide portion of the insulin B chain. This photoaffinity ligand, which also contained a biotin at Lys-B29 and an ^{125}I at Tyr-A14, labeled a single 135-kDa peptide with 60–100% cross-linking efficiency.

Mapping an SH2 Domain by Photoaffinity Scanning. *Src* homology 2 (SH2) domains are general recognition sites for phosphorylated tyrosines, and they play a key role in signal transduction. A 120 amino acid SH2 domain of phosphatidylinositol 3-kinase p85 was mapped using a series of small phosphopeptide photoprobes bearing Bpa substitutions (Williams & Shoelson, 1993). A complete set of photoreactive analogs was prepared in which each amino acid (except phosphotyrosine) was successively replaced by Bpa. In this new approach, termed photoaffinity scanning, the regions of protein–protein interaction could be more effectively mapped. The modified phosphopeptides cross-linked the SH2 domain with high efficiency for three of the Bpa peptides. When the replaced amino acid was adjacent to the phosphotyrosine (Bpa ± 1 analogs), efficient cross-linking was observed at Gln-83. The Bpa + 4 substitution led to regiospecific cross-linking at Asn-85.

Angiotensin (Ang) Receptor Mapping. Two mechanistically different Ang II analogs, [Sar^1,D-Phe(N$_3$)]Ang and [Sar^1,-Bpa^8]Ang, were developed as photoprobes for mapping the Ang receptor subtypes (Bosse et al., 1993). The aryl azido ligand selectively labeled the subtype 1 receptors, while the BP probe did not label this receptor, despite showing activity in binding assays. The selectivity was reversed for labeling the subtype 2 (AT_2) receptors. The BP photoprobe [Sar^1,-Bpa^8]Ang was efficiently photoattached to the human myometrium membrane (AT_2) receptors (70%) and labeled a single 68-kDa band (Servant et al., 1993), while the azido derivative failed to photocovalently modify the binding site. In another approach, an N-terminal BP-acylated Ang II analog ([4-benzoylbenzoyl^1,Ile^8]Ang II) was used successfully to label a single peptide in isolated uterine membranes (Moore et al., 1990).

Atrial Natriuretic Receptor Photolabeling. A highly efficient labeling of the hormone binding domain of atrial natriuretic factor (ANF) receptor was achieved with a model peptide, where the Bpa residue was substituted for Arg-125 of a 99–125 portion of the natural ANF (McNicoll et al., 1992). The Gln-116 residue was also replaced by the iodinatable Tyr which, together with Bpa and the Bpa-adjacent Phe-124, form a hydrophobic core positioning the photoreactive residue deep inside the peptide binding domain. This probe selectively labeled a 130-kDa polypeptide with 64% covalent incorporation, which was 10-fold higher than that attained using azidophenyl probes.

Phototopographical Labeling of Human Luteinizing Hormone Subunits. Bpa can also be used for phototopographical labeling, that is, to identify subunit contact sites (Keutman & Rubin, 1993). Subunit complementary sites were examined in human luteinizing hormone, which is a heterodimer consisting of noncovalently associated α- and β-subunits. An N-terminal portion of β-subunit [hLHβ-(1–15)] was found to inhibit α/β association, while maintaining hormone activity toward its receptor. The isosteric Trp-8 was changed to Bpa, and the resulting protein, [Bpa^8]-hLHβ-(1–15), was photochemically cross-linked to the α-subunit. The site of contact in the α-subunit was located at Met-29 and Gly-30 residues of the N-terminal fragment.

Helical Polypeptides and ATPase. The 26 amino acid melittin, a bee venom toxin, inhibits gastric (H^++K^+) ATPase (Cuppoletti & Malinowska, 1992). A structurally similar 17-residue model peptide (Trp^3), in which seven lysines and eight leucines are arranged to form an amphipathic α-helix, prevented the photolabeling of ATPase with an aryl azide derivative of melittin. Two Bpa-modified Trp^3 analogs were used for photolabeling, and in each one a Lys was replaced by Bpa. Both Bpa-containing analogs labeled the ATPase, resulting in a single cross-linked polypeptide.

PHOTO-CROSS-LINKING WITH HETEROBIFUNCTIONAL CROSS-LINKING REAGENTS

4-Maleimidobenzophenone (MBP). The thiol-reactive bifunctional reagent MBP (**34**, Chart 3) was first prepared and employed in examining the conformational differences between the monomeric and polymeric states of actin (Tao et al., 1985). The approximate length of this benzophenone side chain was 10 Å, and cross-linking served as a molecular ruler to scan distances between peptide chains.

This technique was used to identify the contact sites of the troponin subunits TnC, TnI, and TnT from rabbit skeletal muscle (Leszyk et al., 1988). The MBP-modified TnC (BP-TnC) at Cys-98 forms a binary complex with either TnI or TnT, and irradiation creates a covalent linkage between the subunits in the presence of Ca^{2+}. The dual center of the covalent attachment was at Arg-108 and Pro-110 in the TnI subunit, possibly in a predicted β-turn, which lies close to Cys-98 of TnC. These findings also revealed that Ca^{2+} bound to a region near Cys-98, and the resulting conformational changes of TnC triggered the binding to TnI. The attachment site of the BP-TnC·TnT covalent dimer was similarly identified. Photolabeling of the ternary complex (TnC, TnI, TnT) resulted in identical locations of cross-linking, indicating an absence of competition between TnI and TnT. A mutant TnC (TnC57), in which the thiol functionality was transferred from Cys-98 to position 57, was similarly employed to map the C-helix contact-site region (Wang et al., 1990).

Photochemical cross-linking can provide direct evidence for possible biochemical events. The distance between Cys-133 of TnI and Cys-374 of actin increased by approximately 15 Å on the binding of Ca^{2+} to TnC. In fact, TnI labeled at Cys-133 with MBP could be cross-linked to actin in the absence of Ca^{2+}, but not in its presence. Thus, TnI may act as a Ca^{2+}-dependent molecular switch in the regulation of muscle contraction, being attached to actin in the Ca^{2+}-free (relaxed) state of muscle and detached from actin followed by the activation of contraction by Ca^{2+} (Tao et al., 1990).

Associations between actin (A) and gelsolin (G) were examined by photolabeling using BP-actin conjugating the Cys-374 residue with MBP (Doi et al., 1991). Gelsolin forms an A_2G complex in the presence of Ca^{2+}; however, upon irradiation, a simple AG covalent complex was observed and no cross-linking was detected in the absence of Ca^{2+}. If a preformed A_2G complex was treated with EGTA to remove Ca^{2+} and irradiated, the AG cross-linked adduct was again identified. This tight-binding actin cannot be released after association and has close contact only with gelsolin within a 10-Å distance.

MBP was used to derivatize wheat germ CaM at Cys-97 in the N-terminal domain (Strasburg et al., 1988). This

photoprobe stimulated ATPase in the dark, and upon irradiation it pecifically labeled a single protein in cardiac sarcoplasmic reticulum ATPase. This probe also successfully labeled phosphorylase *b* and two subunits of troponin. Coupling of BP to two thiols in chicken gizzard caldesmon (CaD) gave CaD–BP, which retained CaM and actin binding activity. The CaD–BP probe gave a single cross-linked CaD–actin complex in the absence of Ca^{2+} and two products with CaM, CaD–CaM and CaD–$(CaM)_2$, in the presence of Ca^{2+}. This pattern suggested that both SH groups were proximal to the CaM binding region (Wang, 1988).

Intramolecular cross-linking was employed to examine the effect of nucleotide binding on the environments of the two thiols of myosin subfragment 1 (SF_1), thiol-1 and thiol-2. When thiol-2 was conjugated with MBP, light-induced cross-linking occurred from SF_1-thiol-2-MBP to the central 50-kDa segment of SF_1, but a significant shift of the cross-linking site within the segment was determined upon the addition of MgADP (Rajasekharan et al., 1989). That result indicated a close proximity of the thiol-2 region (Cys-697) to the adenosine binding site. In the absence of nucleotide, the site of the attachment was identified as Arg-239, proximal to the N-terminus (Agarwal et al., 1991).

4,4′-Dimaleimidobenzophenone, a Trifunctional Cross-Linker. In a related approach, 4,4′-dimaleimidobenzophenone (**35**, Chart 3) was employed as a photoactivatable, trifunctional, thiol-trapping reagent to monitor the conformational changes of SF_1 that take place during phosphate binding (Rajasekharan et al., 1987). A BP bridge was established between the thiol-1 and thiol-2 groups and photolytically labeled different regions, depending on whether or not MgADP (or $MgPP_i$) was present in the binding site. With the adenosine moiety, the labeling was highly efficient and site-specific to the 21- and 50-kDa segments.

4-(2-Iodoacetamido)benzophenone (BPIA). An analogous thiol-specific, photoactivatable reagent, 4-(2-iodoacetamido)-benzophenone (BPIA) (**36**, Chart 3), was used to couple BP selectively to thiol-1 (Cys-707) of the myosin SF_1 (Lu & Wong, 1989). Both cross-linked regions were found as a part of the 25-kDa head structure of SF_1. The major site of cross-linking was identified as Glu-88, far from the nucleotide binding pocket; therefore, MgADP binding did not change the conformation around thiol-1, as was found previously. Very recently, BP was linked to phosphorothioate groups in modified oligonucleotides by using BPIA, and this probe effectively labeled tRNA synthetase (Musier-Forsyth & Schimmel, 1994).

Benzophenone-4-isothiocyanate. Interactions between gelsolin and the actin-associated tropomyosin were studied using horse plasma gelsolin, which had been labeled at multiple sites with an amino-reactive heterobifunctional cross-linker, benzophenone-4-isothiocyanate (**37**, Chart 3) (Koepf & Burtnick, 1992). Photolysis of BP–gelsolin in the presence of rabbit cardiac tropomyosin and Ca^{2+} showed a single band, which probably represents a ternary, cross-linked 2:1 complex of tropomyosin:gelsolin.

Other Reagents. An imidate-functionalized BP (**38**) was among the first reagents employed for protein modification (Mariano et al., 1976). The [^{3}H]BZDC reagent (**39**) was discussed in detail in the Biochemical Applications of Tethered Benzophenones section above and was first developed for IP_3R labeling (Mourey et al., 1993).

FUTURE DIRECTIONS

New biochemical applications for the BP photophore have proliferated in the last 5 years. Efficient photoaffinity probes provide gross structural maps that complement more detailed primary, secondary, and tertiary structural information obtained from sequencing and structural biological methods. BP photophores offer simplified handling in ambient light, greater flexibility in the selection of synthetic reaction conditions, higher efficiency of covalent modification, and fewer side reactions with nonproximal residues.

Combination of BP photolabeling with other methods offers many opportunities for creativity. The bidentate photolabeling combines active-site-directed labeling and photo-cross-linking and may provide information about the conformational changes upon binding. Quantitative photoaffinity labeling can be used as a targeting tool to position other functionalities adjacent to the binding site, allowing for further manipulation. Quantitative photocovalent modification can also inactivate enzymes and either activate or inactivate a receptor or binding macromolecule.

A recent review of photolabeling probes summarizes the areas in which rapid development can be expected after certain difficulties are resolved (Brunner, 1993b). One of the major problems identified was how to improve the methods for determination of the cross-linking site. One promising solution suggested was label transfer cross-linking, which results in a disconnection after the photocovalent attachment, leaving only the label at the contact site. The photoactive group is linked to a cleavable arm, which is detached by mild chemical treatment such as ester hydrolysis or disulfide reduction. Electron spray mass spectroscopy and MALDI-MS could also provide excellent alternatives, allowing identification of the modified amino acid contained in a the covalently modified peptide fragment.

Cross-linking and subsequent cleavage might provide a tool for studying signaling systems. One can conceive of covalent photoactivation to an on state, with engineering of a photodeactivation process to produce an off state. The development of a photoswitchable signaling system constitutes an important challenge.

The biosynthesis of photoactivatable proteins is possible by the insertion of unnatural photolabile designer amino acids using modified tRNAs during peptide synthesis (Brunner, 1993a). Such site-specific photoreactive proteins will be selectively targeted to the corresponding membrane or function, allowing study of their interactions at exactly the right location and environment.

Multiplexed biochemical assays have been performed to exploit the precision and high resolution of photolithography, which was combined with light-directed, combinatorial chemical synthesis (Fodor et al., 1993). Biopolymers (oligopeptides or oligonucleotides) are packed into highly compact matrices at densities exceeding 4000/50 mm^2 to give biological chips. Such arrays make it possible to conduct multiple assays in a very short time under highly uniform conditions.

Adaptation of BP photophore technology may offer yet more revolutionary new prospects. Rapid developments in laser extraction, computer, analytical, and automation techniques favor light-induced methods, which can be controlled more closely than those that employ chemical reagents.

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