Optimization and Immunological Characterization of a Photochemically Coupled Lysergic Acid Diethylamide (LSD) Immunogen

Sarah Kerrigan[†] and Donald. E. Brooks*

Departments of Chemistry and Pathology and Laboratory Medicine, 2211 Wesbrook Mall, University of British Columbia, Vancouver, British Columbia, Canada V6T 2B5 . Received March 18, 1998; Revised Manuscript Received June 30, 1998

A photoreactive heterobifunctional linker was used to prepare an immunogen in which lysergic acid diethylamide was indirectly coupled to keyhole limpet hemocyanin at multiple sites on the drug. It was possible to attach approximately 35 drug molecules to each protein using approximately equal amounts of both species during the reaction. The presence of buffer components or water severely compromised reaction efficiency, as estimated from the molar substitution ratio. Factors such as excess linker, pH, irradiation of dry matrix in the absence of buffer, and the drug/protein ratio used during photolysis were shown to have pronounced effects on reaction efficiency. Structural insights regarding immunogen coupling were obtained by determining the specificities of antibodies which were raised against the immunogen. Cross-reactivity data indicated that haptenation of protein likely occurred at positions N1 and N6 of lysergic acid diethylamide, which is plausible given the electrophilicity of the photogenerated aryl nitrene.

INTRODUCTION

The haptenation of protein for the purpose of raising antibodies to small molecules such as drugs or toxins influences the immune response at the T-cell level (*1*). The production of antibody is sterically influenced by the site of attachment between drug and carrier molecule. In practice, this effect is generally observed in cross-reactivity studies as decreased antibody specificity in the vicinity of the linkage. The chemistry of the linker moiety and the hapten density affect both the intensity and the character of the immune response, as does the nature of the carrier molecule (*2*). In theory, a superior antiserum is obtained following immunization with a highly immunogenic conjugate in which drug is attached to the carrier protein via an extended spacer arm to improve specificity. This approach allows the hapten to be held a short distance away from the protein surface, thus increasing the possibility that lymphocytes can distinguish the haptenic determinant at the site of attachment. Immunogen design, therefore, has a profound effect on the affinity, specificity, and overall characteristics of the antiserum. The quality of the antibody reagent can have a pronounced effect on the performance of immunoassays, which are frequently used to determine the concentration of a drug in a biological specimen.

Lysergic acid diethylamide (LSD)[1] is a controlled drug which is abused for its potent psychoactive properties. A typical dose is only between 50 and 100 μg as a result of its unusually high potency, relative to other common hallucinogens such as psilocybin or mescaline. As a result of the small dose ingested and its rapid biotransformation, LSD detection in biological matrixes can be a formidable task for drug detection agencies. Immunoassay methodologies which rely on competitive binding principles require highly sensitive antibodies for successful measurements in the sub-microgram-per-liter region of forensic interest.

There have been surprisingly few developments in LSD immunogen design spanning the history of the drug's use. By far the most successful and widely used technique has been to couple the indole nitrogen of LSD to a carrier protein using a type of Mannich condensation, first described 25 years ago (*3*, *4*). Protein haptenation has also been accomplished via carbodiimide-mediated linkage at the carboxyl group of lysergic acid (*5*, *6*). The incorporation of a carbon spacer between the drug and carrier has been described for chemical linkages at both these reactive sites on the drug molecule (*7*, *8*). At the present time, there are no known reports of LSD immunogens which utilize chemical linkages at sites other than N1 or C8, due to the complex chemistry of the drug and the limited number of reactive sites.

Recently, a new LSD immunogen was prepared using a novel haptenation procedure, whereby drug was coupled to the carrier protein in a nonspecific manner at a number of possible sites (*9*). Polyclonal antibodies raised against this immunogen were used to detect picogram quantities of LSD in urine by enzyme-linked immunosorbent assay (ELISA) (*10*). It is believed that the improved detection limits toward unmetabolized LSD were the results of multisite attachment of drug to carrier protein, which produces a theoretically superior antiserum (*11*). When LSD is coupled to keyhole limpet hemocyanin (KLH) using a photoreactive heterobifunc-

* Author to whom correspondence should be addressed. at the Department of Pathology and Laboratory Medicine. E-mail: don@pathology.ubc.ca. Tel: 604 822 7081. Fax: 604 822 7635.

[†] Department of Chemistry.

[1] BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; LAMPA, lysergic acid methyl-*N*-propylamide; LSD, lysergic acid diethylamide; MSR, molar substitution ratio; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1, 3′-dithiopropionate; TMB, 3,3′,5,5′-tetramethylbenzidine.

S1043-1802(98)00032-9 CCC: $15.00
Published on Web 08/22/1998

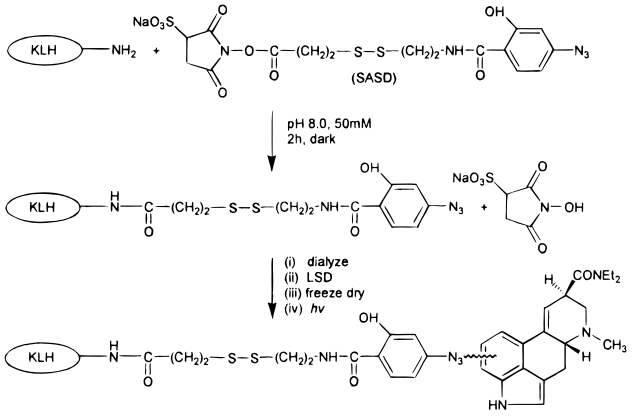

Figure 1. Schematic illustration of immunogen synthesis. The *N*-hydroxysuccinimide ester end of the photoreactive heterobifunctional linker, SASD, was coupled to amine residues on the protein, KLH, by nucleophilic substitution in the dark. After removal of excess hydrolyzed linker and buffer components by dialysis, LSD was added. Derivatized KLH and LSD were freeze-dried and the aryl azide was photoactivated under UV light. After the reaction, excess unbound drug was removed by dialysis.

tional linker, covalent attachments are the result of the relatively nonselective reactivity of the aryl azide moiety. Upon photoactivation, a highly reactive aryl nitrene is produced, which can react with neighboring drug molecules in a number of ways. This approach facilitates multisite attachments at positions on the drug molecule which would not normally be possible using group-specific coupling reagents. This technique reduces the chance that epitopes at the site of attachment are not recognized by the immune system, making more epitopes available for recognition and, therefore, increasing antiserum sensitivity.

Using the heterobifunctional linker sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD), a two-step conjugation procedure was described for the synthesis of the LSD immunogen, KLH-SASD-LSD (*9*). In the first step, primary amines in KLH were coupled to the *N*-hydroxysuccinimide ester end of the linker in the absence of light. Following removal of excess linker, LSD was photochemically coupled to the aryl azide moiety of the modified protein, as outlined schematically in Figure 1.

This work describes a number of variables which were found to affect the conjugation procedure, in particular the overall efficiency of the reaction which was estimated from the molar substitution ratio (MSR). A theoretical discussion of feasible linkages is provided with respect to aryl azides. Finally, immunological characterization of the antiserum in a cross-reactivity study was used to obtain some structural insight into the possible sites of attachment during haptenation.

EXPERIMENTAL SECTION

Materials. Keyhole limpet hemocyanin, sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate, and Slide-A-Lyzer dialysis cassettes were obtained from Pierce (Rockford, IL). Coomassie Brilliant Blue G-250 was supplied by Eastman Kodak (Rochester, NY). Fluorescamine [4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione], dithiothreitol (DTT), 3,3′,5,5′-tetramethylbenzidine (TMB), and goat anti-rabbit IgG peroxidase were obtained from Sigma (St. Louis, MO). ACS grade inorganic salts, glycine, acids, organic solvents, and hydrogen peroxide were supplied from Fisher Scientific (Edmonton, AB). Dimethyl sulfoxide (DMSO) was dried over molecular sieves prior to use.

Iso-LSD and lysergic acid methyl-*N*-propylamide (LAMPA) were supplied by Dr. Kevin Gormley through the National Institute on Drug Abuse (NIDA) Drug Supply Program (Rockville, MD). Dr. Haro Avdovich of the Bureau of Drug Research (BDR), Health Canada, (Ottawa, ON) supplied *d*-lysergic acid diethylamide, dimethyltryptamine, *d*-lysergic acid, and lysergic acid amide. Ergonovine maleate, α-ergocryptine, ergotamine tartarate, lysergol, and *N*-demethyl-LSD (nor-LSD) were purchased from Sigma (St. Louis, MO). Research Biochemicals Inc. (Natick, MA) supplied ergocornine, ergocrystine, methylergonovine maleate, methysergide maleate, and *d*-lysergic acid diallylamide tartarate. Finally, 2-oxo-3-hydroxy-LSD was purchased from Radian International (Austin, TX).

Disposable flat-bottomed 96-well polystyrene microtiter plates were purchased from Corning (New York, NY). A solution of Carnation nonfat skim milk powder (5%) in 150 mM, pH 7.4, phosphate-buffered saline (PBS) was routinely used as the blocking solution in enzyme-linked immunosorbent assays. The TMB substrate solution (*12*), which was prepared immediately before use was made up as follows. Deionized water (16 mL) was added to pH 6.0 acetate/citrate buffer (4 mL) after which 200 μL of 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (10 mg/mL) and 20 μL hydrogen peroxide (3%) were added. The acetate/citrate buffer was made by adding 200 mM citric acid to 200 mM sodium acetate to give a final pH of 6.0. The Bradford dye binding reagent (*13*) was prepared by dissolving Coomassie Brilliant Blue G-250 (10 mg) in 5 mL of ethanol after which 10 mL of phosphoric acid (85%) was added and the total volume made up to 100 mL with deionized water.

Modification of Protein with SASD at Varying pH. The reaction efficiency of SASD with protein at different pH values was estimated by measuring the decrease in the number of amines before and after the reaction. Quantitative determination of primary amine residues was performed using fluorescamine as previously described (*14*). Briefly, a sample containing primary amines (1.5 mL) was vortex mixed with 0.5 mL fluorescamine (0.3 mg/mL) in acetone. After 10 min, the fluorescence was measured using a Turner model 430 Spectrofluorometer at excitation and emission wavelengths 395 and 475 nm, respectively. Glycine was routinely used as the standard to compensate for changes in fluorescence intensity over a range of pH.

It was necessary to remove the photoreactive aryl azide group of the derivatized protein prior to the fluorescamine assay. This was achieved by cleavage of the disulfide group using the reducing agent dithiothreitol (DTT). The percent of protein residues which were modified with SASD over a range of pH was measured using BSA in place of KLH, to minimize solubility problems. In the absence of light, 100 μg of BSA was reacted with SASD (0.14 mg in dry DMSO) for 2 h in 50 mM phosphate or carbonate buffer between pH 7.0 and 9.5. Samples containing BSA in the absence of SASD were run in parallel for use as the blanks. DTT (0.06 mg in deionized water) was added to each sample and reacted for 1 h, after which excess linker and the photoreactive end were removed by centrifugal filtration (Centricon-10, Amicon, Oakville, ON). After isolation of the high molecular weight fraction of interest, the number of lysines which were underivatized was estimated from the ratio of fluorescence measurements before and after reaction at

different pH values. The percent of amines that were coupled to SASD was calculated by difference.

Derivatization of KLH with SASD. The *N*-hydroxysuccinimide ester of SASD was coupled to the carrier protein KLH by nucleophilic substitution at optimal pH. An improvement in yield was observed when the first conjugation step was performed directly in the dialysis cassette. This obviates the need for sample transfer after the reaction. The same cassette was rinsed and reused for dialysis in the second conjugation step to reduce additional sample loss due to adsorption on the membrane.

All steps preceding photolysis were performed in complete darkness or in the presence of a red safety light. SASD (5.0 mg) was dissolved in 50 μL of DMSO which had been dried over molecular sieves. KLH (2.0 mg) dissolved in 1.5 mL of 100 mM, pH 8.0, phosphate buffer was injected into a 3 mL Side-A-Lyzer dialysis cassette. SASD in DMSO was injected through a second port into the cassette with gentle agitation. A small air bubble (0.5 mL) was injected to facilitate mixing, and the cassette was taped to an oscillating table and allowed to react for 2 h in the dark. The cassette was then mounted on a floatation device and dialyzed against deionized water or PBS in the dark for 2.5 h with half-hourly water changes to remove the excess unreacted linker or its hydrolysis products. The high molecular weight solution containing the modified protein was removed from the dialysis cassette and stored in the dark until use. LSD (2.2 mg) was added and the mixture was freeze-dried in the absence of light prior to the photoactivation step.

Photoactivation with LSD. KLH which had been derivatized with SASD was photoactivated with LSD using different conditions. Due to the nonselective nature of the photocoupling reaction, the molar ratio of LSD to KLH during the reaction was investigated. The reaction was performed using modified KLH and LSD which had been freeze-dried in the absence of light. The photocoupling step was also performed in solution, in the presence of either water or buffer (PBS). Photoactivation was performed using a Chromato-Vue C3 viewing box (UltraViolet Products Inc., San Gabriel, CA). Samples were placed 10 cm from two long wave (General Electric G15T8, 15W) germicidal lamps and two short wave (Sylvania Blacklite F15T8-BL, 15W) phosphor-coated lamps, which emit light at 254 and 365 nm, respectively. A reaction time of 20 min was sufficient for photoactivation of the aryl azide of SASD, as determined by the 45% decrease in the absorption maximum (*10*) in accordance with the literature (*15*−*17*). Unbound LSD was removed by extensive dialysis over 24 h in a Slide-A-Lyzer cassette. TLC of the purified immunogen indicated that noncovalent incorporation of LSD into the protein was negligible.

Estimation of the Molar Substitution Ratio (MSR). Concentrations of LSD and KLH in the antigen were estimated using spectrofluorometry and colorimetric protein assays, respectively, as previously described (*9*). The purified immunogen was poorly soluble so antigen preparations were allowed to stand overnight at 4 °C, after which the clear supernatant layer containing the soluble fraction was removed for analysis. Quantitative estimates of LSD in the immunogen were based on the assumption that there was no change in the native fluorescence of LSD before and after reaction. The fluorescence spectrum of the soluble immunogen indicated the excitation and emission wavelengths were unchanged (λ_{ex} = 322 nm, λ_{em} = 412 nm), although it is not known how the intensity of the LSD fluorescence is affected once the drug was bound. However, there was a good correlation between this method and the more widely used UV absorption method as demonstrated previously (*3, 9*). The drug content, therefore, was estimated directly from the spectrofluorometric calibration of LSD. The concentration of KLH in the soluble antigen was estimated by the Bradford method (*13*) in which dye-binding reagent (200 μL) and sample (100 μL) were mixed in a microtiter plate. The absorbance was measured using an SLT microtiter plate reader EAR 400 AT (SLT-Labinstruments, Austria) at 550 nm and the concentration of KLH in the immunogen was interpolated directly from the calibration graph.

Investigation of Immunogen Structure. Structural insights regarding the sites at which LSD was coupled to KLH in the immunogen were obtained by measuring the cross-reactivity of the polyclonal antiserum produced in rabbits which were immunized with the conjugate. A standard immunization protocol was used whereby 150 μg of immunogen was injected subcutaneously in Freunds complete adjuvant followed by intramuscular booster injections in Freunds incomplete adjuvant at monthly intervals (*18*). The specificity of the antiserum toward a number of structurally related analogues to LSD was measured using a competitive binding ELISA (*10*). Polystyrene microtiter plates were coated overnight at 4 °C with 100 μL BSA−LSD conjugate (10 mg/L in PBS) prepared by the Mannich reaction (*3, 4*). After treating uncoated sites on the well with 150 μL of blocking solution (5% skim milk powder in PBS) for 30 min at 37 °C, wells were washed thoroughly with PBS. LSD or related compounds were diluted in blocking solution (10^{-7}−10^{-12} M), and 50 μL was added to the plate. An additional 50 μL of rabbit anti-LSD in blocking solution was added, such that the overall serum dilution was 1:50. After incubation for 2.5 h at 37 °C, the plate was rinsed with PBS and 100 μL goat anti-rabbit IgG peroxidase (1:1000) in blocking solution was added. Following a 30 min incubation at 37 °C, wells were washed with PBS and 100 μL TMB substrate solution was added. After 5 min, the reaction was stopped using 50 μL and 1 M sulfuric acid and the absorbance was measured at 450 nm using 620 nm as the reference using a microtiter plate reader.

RESULTS AND DISCUSSION

Derivatization of Protein with Linker. The incorporation of sufficient LSD for immune recognition is dependent on the successful modification of KLH with aryl azides. Only the unprotonated amines on the protein are reactive toward the NHS ester region of the linker; therefore, it is necessary to use a pH at which a significant number of amines are uncharged. Fluorescamine was used to determine the pH conditions at which the availability of unprotonated amines was maximized (Figure 2). A shift in pH from neutral to alkaline pH increased fluorescence, which is linearly related to the number of primary amines, more than 3-fold. This is in accordance with the deprotonation of lysine residues (pK_a $\approx$ 10) which is the principal source of primary amines in the protein.

Fluorescence measurements before and after reaction were used to estimate the number of lysines which had been derivatized with SASD at different pH values. Figure 2 illustrates the delicate balance between competing aminolysis and hydrolysis reactions. Increasing stoichiometry with increasing pH is the result of deprotonation of amine groups whereas the decrease in reac-

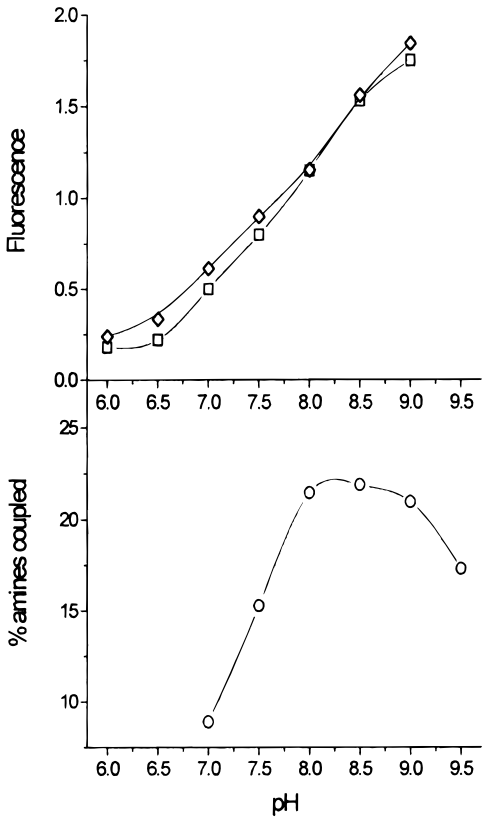

Figure 2. Effect of pH on availability of primary amines using the fluorescamine assay. BSA, 6.25 μg (diamonds), KLH, 25 μg (squares). The fluorescence signal, which is linearly related to the number of amines, was calculated relative to glycine, at the same pH. (Below) Effect of pH on reaction efficiency using BSA and SASD (circles). The percentage of amines coupled to linker was determined by difference using fluorescamine, before and after reaction.

tion efficiency at elevated pH is the result of NHS ester hydrolysis. A shift of 1 pH unit from 7.0 to 8.0 increased the derivatization of lysines by more than a factor of 2. Therefore, a pH of 8.0 was chosen as a compromise between the number of available amines and the lability of the ester. The ester moiety of SASD although stable at acid pH, hydrolyzes to *p*-azidosalicylamido-1,3′-dithiopropionate and *N*-hydroxysuccinimide (NHS) under alkaline conditions. The half-life of NHS-esters at pH 9, 7.5, and 5 is reported to be 1, 14, and >90 min, respectively (*19*). Optimum substitution is achieved when a large molar excess of linker is used to overcome inefficiencies due to hydrolysis (*20, 21*). When a 20 molar excess of linker to primary amines on KLH was used at pH 8.0, approximately 45% of the lysine residues were coupled to SASD. Assuming there to be about 689 lysine residues in the KLH molecule, this corresponds to a total of about 310 photoreactive linker moieties attached to each protein, assuming a molecular mass of 3×10^6 Da (*9, 22*).

Photochemical Coupling and the Molar Substitution Ratio. Estimation of the molar substitution ratio using LSD fluorescence and protein quantitation provided useful insight into the effect of photocoupling conditions on reaction efficiency. Most of the typical methods used for determining substitution ratios could not be used due to the very large size of KLH [350−13 000 kDa (*23*)] and the poor solubility of the immunogen (~5 μg/mL). UV spectroscopic determination was not possible due to the overlapping absorption of SASD with both KLH and LSD. Indirect spectroscopic techniques, such as the use of trinitrobenzene sulfonic acid (TNBS) allow the colorimetric determination of unreacted amino groups in the milligram per milliliter concentration range. Radiolabeled haptens can be used to measure the extent of coupling directly, by measuring the associated radioactivity of the conjugate. However, this technique is not advocated for photochemical coupling due to non specific ("dark") labeling or photodeiodination (*15, 24*). Mass spectrometry, particularly electrospray and matrix-assisted laser desorption ionization techniques, have become increasingly useful for the characterization of conjugates which are soluble at sufficient (approximate micromolar) concentration (*25*−*27*).

Characterization of the LSD immunogen in which relatively few drug molecules are attached to a protein with a molecular mass of several million is not an easy task. The fluorescence technique was the only method suitable for sensitive and specific estimation of LSD bound to KLH in the immunogen (*9*).

The combined fluorescence and Bradford dye-binding methods provides only a comparative approximation of the LSD content in the soluble immunogen fraction since it is dependent on a number of assumptions. The photolytic approach to immunogen synthesis likely results in a number of high molecular weight products with varying degrees of substitution. The first assumption is that the LSD content of the soluble fraction is representative of the entire sample, which might not be true. Solubility is likely to decrease with increasing substitution of protein which might result in the least soluble complexes (which have higher MSRs) precipitating out.

Optimization of Photocoupling Conditions. As expected, removal of excess unreacted linker prior to photoactivation was essential. Its presence in the reaction mixture severely compromised reaction efficiency by competing with drug for the limited number of aryl azide moieties. Liquid-phase photoactivation also decreased the MSR of the immunogen. When photolysis was performed in aqueous solution rather than on a freeze-dried sample, there was more than a 40% decrease in efficiency. Removal of water from the reaction mixture is preferred because the nitrene can react with water to form hydroxylamine (*28*), which decreases the substitution ratio. Despite reports of poor reaction efficiencies due to photolysis in aqueous solvents (*16*), there are no known reports in the literature of conjugation by dry photoactivation, as was routinely employed in this study.

Molar substitution ratios were measured over 2 orders of magnitude using dry photoactivation under various conditions. The covalent coupling of aryl azides on KLH with neighboring molecules is nonspecific. Therefore, to increase the chance of reaction with LSD, a large excess of drug was used. Figure 3 summarizes the results of 12 immunogen preparations performed over a number of weeks in which the molar ratio of LSD to KLH employed during photoactivation was varied. Dried down buffer components were present in the reaction matrix during irradiation. Clearly, the initial LSD/KLH ratio employed during photolysis determined the degree of substitution in the immunogen. There was a good correlation between increased concentration of LSD during photolysis and increasing molar substitution ratio ($r = 0.958$). The use of large excesses of drug during photolysis is a definite drawback, however, considering that LSD is a controlled substance which is available in limited supply. Reaction conditions which produced optimum substitution under these conditions could not

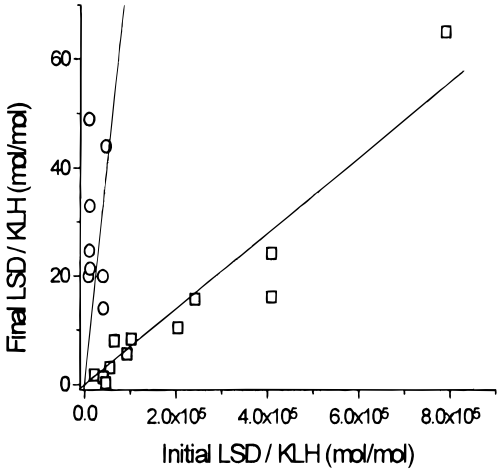

Figure 3. Effect of excess LSD on molar substitution ratio (MSR). Immunogen was prepared by irradiation of freeze-dried modified protein and LSD in the presence of dried buffer components (squares) and in the absence of buffer (circles).

be employed on a preparative scale for immunization of rabbits, because the quantity of drug required was too large.

However, dialysis of the immunogen matrix against deionized water prior to photoactivation showed improved performance (Figure 3). A dramatic increase in reaction efficiency was observed when buffer components were removed prior to irradiation. By this method, molar substitution ratios of 20−50 were attainable using only slight excesses of drug. This suggests that buffer components in the reaction matrix compete with LSD for the limited number of reactive nitrenes on the protein. In the absence of buffer, photoactivation of approximately equal amounts of KLH and LSD by weight produced conjugates with average substitution ratios of about 35.

Reaction Efficiency. The efficiency of the reaction was calculated from the percentage of lysine residues on KLH that were eventually coupled to LSD. The average two-step reaction efficiency for the injected immunogen was 5% assuming 35 LSDs attached to each KLH molecule, which contains about 689 lysine residues/3 × 10^6 Da molecule (*9*). It was estimated that only about 45% of the total lysines on KLH were derivatized with SASD. Therefore, the reaction efficiency of the photolysis step toward LSD using optimized conditions was approximately 11%, which is quite favorable considering the nonselective nature of the photochemical coupling. This is likely the result of solvent and buffer removal prior to photolysis and the presence of nucleophilic groups on LSD which attract the electrophilic nitrene. Typical efficiencies of photoreactive linkers which are used to couple high molecular weight species are generally low due to the nonspecific nature of the reaction. Reaction efficiencies of 15% using conventional aryl azide photoprobes such as SASD are considered good (*29*).

Reaction efficiencies vary greatly, depending on the type of linker used. The recent introduction of polyfluorinated phenyl azide heterobifunctional linkers, which generate strongly electrophilic nitrenes, result in greater efficiencies (*30*, *31*) typically reported between 20 and 60% (*32*). The use of a fluorinated photoreactive linker and a carrier protein which contains more lysine residues would improve efficiency. The availability of primary amines on KLH and BSA at pH 8.0 was determined using the fluorescamine assay. Following linear regression analysis, a direct comparison of the slopes for BSA (0.233 ± 0.009, $r = 0.997$) and KLH (0.057 ± 0.004, $r = 0.987$) indicated that KLH had only 25 ± 3% of the lysines available on BSA by weight. This finding suggests that overall higher reaction efficiencies might be achieved using albumin, although the immunogenicity of the derivatized BSA conjugate may be lower than that of KLH.

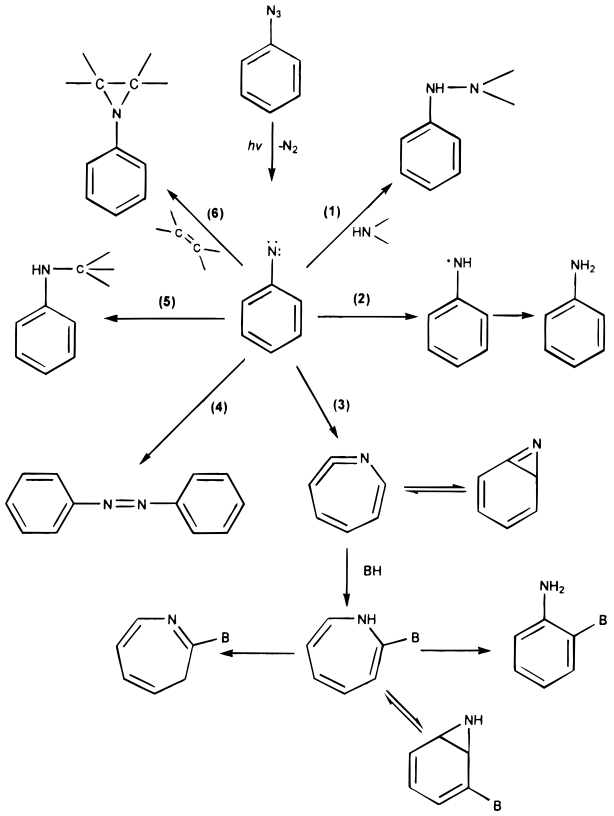

Figure 4. Important reactions of the aryl azide group.

Theoretical Considerations. Insertion of aryl azides in model systems such as cyclohexane or diethylamine is well documented (*33*), although investigation of protein conjugates is rarely attempted. The relationship between insertion characteristics of the nitrene in a model system compared to a macromolecule is extraordinarily difficult due to the variety of functional groups and environments in the protein (*16*). The complex photochemistry of aryl azides involves three excited states and at least six short-lived ground-state intermediates. Photolysis of phenyl azides usually results in multiple reaction pathways of highly amorphous products which make interpretation and identification often difficult. To complicate things further, the products are highly variable, depending on reaction conditions such as an inert or reactive solvent systems, characteristics of the light source and substituent groups on the aryl azide ring. To gain some insight into the reactivity of the derivatized protein toward neighboring molecules, some important reactions of aryl azides are summarized in Figure 4. These include insertion into NH bonds to form hydrazines (**1**), reduction of the azido group (**2**), nucleophilic ring expansion to form azepines (**3**), formation of azobenzene (**4**), insertion into CH bonds (**5**), and addition across double bonds to form aziridines (**6**).

It should be noted, however, that the most favorable reaction pathways are primarily dependent on the electrophilic character of the nitrene and its environment. CH insertion products are produced using very strongly electrophilic nitrenes, such as polyfluorinated phenyl azides. Strong electron-withdrawing groups increase the

reactivity of the nitrene toward CH bond insertion. Aryl azides without such substituents are more likely to react with other groups, such as nucleophiles, especially if they are embedded in a nucleophilic environment, such as a protein molecule.

The lifetime of a reactive nitrene is about 10^{-4} s (*34*), although this depends on the substituent groups attached to the aryl ring (*35*). Aryl nitrenes or their intermediates may react with C−H, N−H, and aryl groups, which are present in the LSD molecule. The nitrene is one of the few reactive species that can react with C−H by abstraction and insertion, therefore allowing covalent attachment in the absence of any particular functional group. Because the species is particularly reactive toward nucleophilic groups, it is likely that a large part of the nitrene reactivity on the modified protein will be self-directed. However, the 19 Å spacer arm of SASD increases the likelihood of a bimolecular reaction (*35*).

An inherent disadvantage of the photocoupling technique is its low efficiency compared to most site-specific bioconjugation techniques. Yields resulting from photoreactive linking of unsubstituted aryl azides are generally low, and efficiencies less than 10% are considered acceptable (*20*). Efficient coupling requires not only a reactive nitrene but also a favorable orientation toward the target molecule. To partly compensate for this, it is necessary to use a large excess of LSD to improve the probability that the photogenerated species is in proximity to a drug molecule.

Perhaps the most important difference between the literature reports of aryl nitrene chemistry and the system we describe is the use of a solid matrix for photoactivation. There have been only a few reports of the effect of temperature and phase on aromatic nitrene chemistry. Although the simple systems examined do not resemble our immunogen reaction matrix, a number of interesting observations have been made. In a restricted environment, such as solid state or ligand bound to macromolecule, radical coupling may predominate due to limited movement of the reactive species (*36*). It was suggested that the increase in CH insertion products via radical recombination in a "rigid" matrix was due to trapping aryl azides in the hydrophobic crevices of the protein, which resulted in higher protein labeling efficiencies (*16*).

Photoactivation of the aryl azides on KLH in the presence of LSD likely results in a great many products given the complexity of the reaction matrix and the diversity of functional groups which are present. A mixture of both favorable and unfavorable reaction products are contained in the high molecular weight fraction.

The most desirable reactions are those which take place between modified KLH and LSD, of which there are a number of hypothetical products (Figure 4). Azepine type conjugates (**3**) may result from the reaction between the electrophilic nitrene and nucleophilic substituents on LSD. Lone pairs on amino nitrogen and other nucleophiles are known to attract the nitrene. In LSD, the nitrogen in the 6 position is strongly basic due to the presence of three electron-donating alkyl groups. The indole nitrogen, which is less basic due to the aromaticity of the indole ring, could also react with a nitrene to form an azepine. Addition across double bonds (**6**) could also occur at a number of sites on the LSD molecule, although there are very few reports of stable aziridine products from such reactions (*28*). Singlet nitrenes can insert into CH bonds (**5**) with varying efficiencies depending on the CH bond strength. A possible site for this reaction could be the acidic hydrogen in the 2 position, which would lead to formation of an amine. Phenyl azides are also capable of NH insertion reactions (**1**) which could take place at the indole nitrogen to produce a hydrazine product. In summary, there are a number of hypothetical reaction sites on LSD which would result in covalent attachment of the drug to the modified protein.

However, there are many undesirable reactions which might occur in the reaction matrix between aryl azide and neighboring amino acid residues. The reactivity of aryl nitrenes toward functional groups found in biological molecules is unquestionable. In particular, reactivity with aryl (Phe, Tyr, Trp, His), N−H (Lys, His, *N*-termini), O−H (Ser, Thr, Try), and S−H (Cys) has been suggested (*37*). In one report, photolysis of [^{125}I]SASD in the presence of BSA indicated incorporation of radiolabel into His, Thr, Lys, Cys, Glu, and Asp (*38*). It is quite likely that the bulk of the nitrene reactivity is directed toward these residues and not the target molecule, LSD. For this reason, it is necessary to use an excess of drug during photolysis, so that LSD is utilized as a "scavenger". These species are used in conventional photoaffinity labeling to preferentially react with photogenerated intermediates at places other than the ligand-binding site between protein and receptor. Nucleophilic molecules such as tris and *p*-aminobenzoic acid have been used for this purpose (*35*). In a sense, LSD which contains a number of groups which are reactive toward electrophilic nitrenes, is utilized as a scavenger to bind nonspecifically with photolabeled protein.

Poor efficiencies of photoreactive conjugation processes have been attributed to the unwanted reactions which occur between the aryl azide and water or buffer components in the mixture (*35*). Other unwanted reactions involve the formation of aniline (**2**) or azobenzene (**4**) type products. With respect to the latter case, the reaction between two aryl azide moieties in the immunogen is unlikely due to the limited number of reactive nitrenes and the restricted mobility of the derivatized protein in the solid-phase matrix. However, intramolecular cross-linking may occur if the two aryl azide groups are in close enough proximity.

Antibody Specificity and Immunogen Structure. The cross-reactivity of anti-LSD antisera raised against the photocoupled immunogen was measured by ELISA. Regions of the drug molecule which demonstrate reduced specificity indicate that molecular transformation or conjugation may have taken place. An antibody will not recognize a particular epitope if it has not been exposed to it during immunization. This occlusion may occur as a result of the chemical linkage which exists between the hapten and carrier. Depending on the size of the protein and the distance between it and the hapten, areas surrounding the point of attachment may be obscured from the immune system. This is of particular importance for small molecules such as drugs of abuse, for which immunogen design plays a fundamental role in determining the overall antibody specificity (*39*−*41*). As a result, cross-reactivity studies can provide valuable insights into possible structural attachments which have taken place in the immunogen.

Antiserum cross-reactivity toward LSD analogues is illustrated in Figure 5. The approximate percent cross-reactivity was estimated from the concentration of analogue, which produced a signal equivalent to 1.55 nM LSD (0.5 ng/mL), which is the generally accepted cutoff concentration for LSD by immunoassay. The highest cross-reactivity was toward BSA-LSD coupled via the indole nitrogen (100%), 6-nor-LSD (52%) and LAMPA

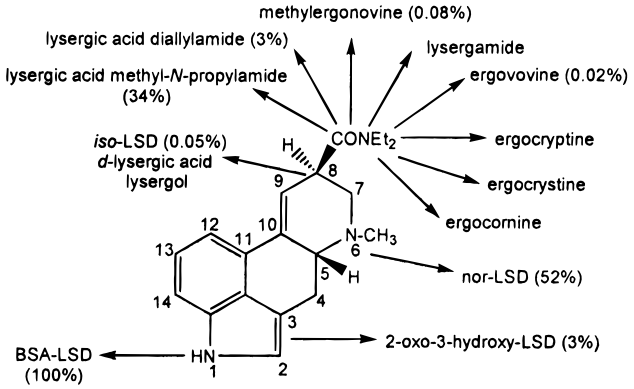

Figure 5. The specificity of the antibody raised against the immunogen was measured by ELISA. The approximate percent cross-reactivity was calculated from the concentration of analogue which produced a signal equivalent to 1.5 nM LSD. Measured cross-reactivities of 0.08% or greater are shown in parentheses.

(34%). In LAMPA, the two ethyl substituents on the amide side chain of LSD are replaced with propyl and methyl groups which do not significantly change the electronic properties of the drug. It is, therefore, no surprise that LAMPA cross-reacts with the antiserum to such an extent. A number of other compounds tested had structural differences at the amide position. When ethyl groups were replaced with hydrogen, in lysergic acid amide, no recognition occurred. In the same way, α-ergocryptine, ergocornine, ergocrystine, ergonovine, ergotamine, and methylergonovine, all of which are modified with large hydrocarbon groups at the amide position, do not significantly cross-react with the antiserum. When C_2H_5 is replaced with $CH_2-CH=CH_2$ in lysergic acid diallylamide, only minimal cross-reactivity was observed (<3%). The replacement of electron-donating ethyl groups with electron-withdrawing allyl groups makes the nitrogen less basic and significantly changes the properties of the molecule in such a way as to prevent effective antibody recognition.

Further insight is provided by analogues which differ from LSD at the C8 position. Replacement of the amide substituent with carboxylic acid or alcohol in *d*-lysergic acid and lysergol produced no cross-reaction and *iso*-LSD, which is a stereoisomer of LSD, cross-reacted only 0.05%. The antiserum recognizes the change in spatial arrangement of diethylamide in *iso*-LSD, thus producing a poor fit between the antibody-binding site and the drug. Overall, the antiserum exhibits good specificity in the vicinity of C8 and the amide. This suggests that, not only did conjugation not occur here but an epitope exists in this region.

Significant cross-reactivity was observed with nor-LSD which is modified in the N6 position. However, it has been shown that replacement of alkyl groups with hydrogen in the amide substituent sufficiently changed the properties of the drug, preventing effective antibody recognition. This not being the case in the N6 region might be suggestive of a covalent attachment having taken place. The reactive nitrene tends to be electrophilic in nature, thus having a preference for regions of high electron density, such as double bonds or lone pairs (*28*). The nitrogen at the 6 position is a tertiary amine, with three electron-donating groups. It is feasible that the electron-deficient nitrene is attracted to the lone pair on this basic nitrogen which subsequently results in covalent attachment at this point. Further evidence for the proposed reactivity between the aryl azide and nitrogen is provided by the cross-reactivity of antibody with BSA-LSD, in which LSD is derivatized at the indole nitrogen (*3*). The indole nitrogen is less basic than the tertiary amine because of the electron-withdrawing double bonds of the indole ring. However, if the nitrene was attracted to the lone pair of electrons on the indole nitrogen, the subsequent chemical linkage would explain the failure of the antibody to distinguish the derivative from the parent drug. In addition to this, the hydrogen atom adjacent to the indole nitrogen is reactive. The removal of this acidic hydrogen results in a nucleophilic carbon in the 2 position which could also attract an electrophilic nitrene. A small cross-reactivity (3%) toward 2-oxo-3-hydroxy-LSD was observed. However, given the likelihood of an attachment at the indole nitrogen, it is possible that this cross-reaction is the result of the nearby linkage which depreciates immune recognition of the drug at adjacent positions.

Structural attachments within the immunogen are almost impossible to determine by conventional means due to the size and poor solubility of the immunogen. Therefore, the cross-reactivity of LSD-like substances provided valuable insight into possible sites of attachment in the immunogen. These data suggest that multisite attachment did occur between LSD and KLH, most likely at positions 1 and 6 of the drug, which is plausible given the reactivity of the photogenerated nitrene.

ACKNOWLEDGMENT

This work was supported by the Medical Research Council of Canada (Grant number MT5759 to D.E.B.).

LITERATURE CITED

(1) Delmas, A., Brack, A., and Trudelle, Y. (1992) Studies of the Influence of Different Cross-linking Reagents on the Immune Response against a B-Epitope. *Bioconjugate Chem. 3*, 80−84.

(2) Klaus, G. G. B., and Cross, A. M. (1974) The Influence of Epitope Density on the Immunological Properties of Hapten-Protein Conjugates. *Cell. Immunol. 14*, 226−241.

(3) Taunton-Rigby, A., Sher, S. E., and Kelley, P. R. (1973) Lysergic Acid Diethylamide: Radioimmunoassay. *Science 181*, 165−166.

(4) Twitchett, P. J., Fletcher, S. M., Sullivan, A. T., and Moffat, A. C. (1978) Analysis of LSD in Human Body Fluids By High-Performance Liquid Chromatography, Fluorescence Spectroscopy and Radioimmunoassay. *J. Chromatogr. 150*, 73−84.

(5) Loeffler, L. J., and Pierce, J. V. (1973) Radioimmunoassay for Lysergide (LSD) in Illicit Drugs and Biological Fluids. *J. Pharm. Sci. 62* (11), 1817−1820.

(6) Van Vunakis, H., Farrow, T. T., Gjika, H. B., and Levine, L. (1971) Specificity of the Antibody Receptor Site to *d*-Lysergamide: Model of a Physiological Receptor for Lysergic Acid Diethylamide. *Proc. Natl. Acad. Sci. U.S.A. 68* (7), 1483−1487.

(7) Ratcliffe, W. A., Fletcher, S. M., Moffat, A. C., Ratcliffe, J. G., Harland, W. A., and Levitt, T. E. (1977) Radioimmunoassay of Lysergic Acid Diethylamide (LSD) in Serum and Urine by Using Antisera of Different Specificities. *Clin. Chem. 23* (2), 169−174.

(8) McNally, A. J., Goc-Szkutnicka, K., Li, Z., Pilcher, I., Polakowski, S., and Salamone, S. J. (1996) An OnLine Immunoassay for LSD: Comparison with GC-MS and the Abuscreen RIA. *J. Anal. Toxicol. 20*, 404−408.

(9) Kerrigan, S. (1997) Immunochemical Detection of Lysergic Acid Diethylamide Using A Photochemically Linked Immunogen. Ph.D. Thesis, The University of British Columbia, Vancouver, Canada.

(10) Kerrigan, S., and Brooks, D. E. (1998) Indirect Enzyme-Linked Immunosorbent Assay for the Quantitative Estimation of Lysergic Acid Diethylamine in Urine. *Clin. Chem. 44*, 985−990.
(11) Strahilevitz, M. (1986) Treatment Methods, Particularly for Psychoactive Drug Dependence. United States Patent Number 4,620,977.
(12) Bos, E. S., van der Doelen, A. A., van Rooy, N., and Schuurs, A. H. W. M. (1981) 3,3′-5,5′-Tetramethylbenzidine as a Ames Test Negative Chromogen for Horseradish Peroxidase in Enzyme Immunoassay. *J. Immunoassay 2* (3/4), 187−204.
(13) Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein utilizing the Principle of Protein Dye Binding. *Anal. Biochem. 72*, 248−254.
(14) Stocks, S. J., Jones, J. M., Ramey, C. W., and Brooks, D. E. (1986) A Fluorometric Assay of the Degree of Modification of Protein Primary Amines with Polyethylene Glycol. *Anal. Biochem. 154*, 232−234.
(15) Koch, T., Suenson, E., Korsholm, B., Henriksen, U., and Buchardt, O. (1994) Pitfalls in Characterization of Protein Interactions Using Radioiodinated Cross-linking Reagents. Preparation and Testing of Novel Photochemical 125I-Label Transfer Reagent. *Bioconjugate Chem. 5*, 205−212.
(16) Pandurangi, R. S., Karra, S. R., Kuntz, R. R., and Volkert, W. A. (1995) High Efficiency Photolabeling of Human Serum Albumin and Human gamma-globulin with [^{14}C]Methyl 4-azide-2,3,5,6-tetrafluorobenzoate. *Bioconjugate Chem. 6*, 630−634.
(17) Wollenweber, H. W., and Morrison, D. C. (1985) Synthesis and Biochemical Characterization of a photoactivatable Iodinatable, Cleavable Bacterial Lipopolysaccharide Derivative. *J. Biol. Chem. 260* (28), 15068−15074.
(18) Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp 53−139, Cold Spring Harbour Laborarory, Plainview, NY.
(19) Carlsson, J., Drevin, H., and Axen, R. (1978) Protein Thiolation and Reversible Protein−Protein Conjugation. *N*-Succinimidyl 3-(2-Pyridyldithio)propionate, A new Heterobifunctional Reagent. *Biochem. J. 173*, 723−737.
(20) Mattson, G., Conklin, E., Desai, S., Nielander, G., Savage, M. D., and Morgensen, S. (1993) A Practical Approach to Cross-linking. *Mol. Biol. Rep. 17*, 167−183.
(21) De-Villiers, M., Parkin, D., Van Jaarrsveld, P., and Van der Walt, B. (1987) A Radioimmunoassay for Metoclopramide. *J. Immunol. Methods 103*, 33−39.
(22) Shuler, K. R., Dunham, R. G., and Kanda, P. (1992) A Simplified Method for Determination of Peptide−Protein Molar Ratios Using Amino Acid Analysis. *J. Immunol. Methods 156*, 137−149.
(23) van Brugen, E. F. J. (1980) Hemocyanin: the mystery of blue blood. *Trends Biochem. Sci. 5*, 185−188.
(24) Cai, S., Glenn, D. J., and Keana, J. F. W. (1992) Toward the Development of Radiolabeled Fluorophenyl Azide-Based Photolabaling Reagents: Synthesis and Photoloysis of Iodinated 4-Azidoperfluorobenzoates and 4-Azido-3,5,6-trifluorobenzoates. *J. Org. Chem. 57*, 1299−1304.
(25) Adamczyk, M., Buko, A., Chen, Y., Fishpaugh, J. R., Gebler, J. C., and Johnson, D. D. (1994) Characterization of Protein-Hapten Conjugates. 1. Matrix Assisted Laser Desorption Ionization Mass Spectrometry of BSA-Hapten Conjugates and Comparison with Other Characterization Methods. *Bioconjugate Chem. 5*, 631−635.
(26) Straub, K. M., and Levy, M. J. (1994) Characterization of Hapten Binding to Immunoconjugates by Electrospray Ionization Mass Spectrometry. *Bioconjugate Chem. 5*, 194−198.
(27) Adamczyk, M., Gebler, J. C., and Mattingly, P. G. (1996) Characterization of Protein-Hapten Conjugates.. Electrospray Mass Spectrometry of Bovine Serum Albumin-Hapten Conjugates. *Bioconjugate Chem. 7*, 475−481.
(28) Patai, S. (1971) *The Chemistry of the Azido Group*, Interscience, New York.
(29) Thevenin, B. J. M., Shahrokh, Z., Williard, R. L., Fujimoto, E. K., Kang, J. J., Ikemoto, N., and Shohet, S. B. (1992) A Novel Photoactivatable Cross-linker for the Functionally-Directed Region-Specific Fluorescent Labeling of Proteins. *Eur. J. Biochem. 206*, 471−477.
(30) Brinkley, M. (1992) A Brief Survey of Methods for Preparing Protein Conjugates with Dyes, Haptens, and Cross-linking Reagents. *Bioconjugate Chem. 3*, 2−13.
(31) Yan, M., Cai, S. X., Wybourne, M. N., and Keana, F. W. (1994) *N*-Hydroxysuccinimide Ester Functionalized Perfluorophenyl Azides as Novel Photoactive Heterobifunctional Cross-Linking Reagents. The Covalent Immobilization of Biomolecules to Polymer Surfaces. *Bioconjugate Chem. 5*, 151−157.
(32) Kym, P. R., Carlson, K. E., and Katzenellenbogen, J. A. (1995) Evaluation of a Highly Efficient Aryl Azide Photoaffinity Labeling Reagent for the Progesterone Receptor. *Bioconjugate Chem. 6*, 115−122.
(33) Schrock, A. K., and Schuster, G. B. (1984) Photochemistry of Phenyl Azide: Chemical Properties of the Transient Intermediates. *J. Am. Chem. Soc. 106*, 5228−5234.
(34) Wilson, D. F., Miyata, Y., Ericiniska, M., and Vanderkooi, J. M. (1975) An Aryl Azide Suitable for Photoaffinity Labeling of Amine Groups in Proteins. *Arch. Biochem. Biophys. 171*, 104−107.
(35) Das, M., and Fox, C. F. (1979) Chemical Cross-linking in Biology. *Annu. Rev. Biophys. Bioeng. 8*, 165−193.
(36) Keana, J. F. W., and Cai, S. (1990) New Reagents for Photoaffinity Labeling: Synthesis and Photolysis of Functionalized Perfluorophenyl Azides. *J. Org. Chem. 55*, 3640−3647.
(37) Bayley, H., and Staros, J. V. (1984) Photoaffinity Labeling and Related Techniques. *Azides and Nitrenes. Reactivity and Utility* (Scriven, E. F. V. Ed.) pp 439−460, Academic Press, Orlando.
(38) Ueno, H., Masuko, T., Wang, J., and Hashimoto, Y. (1994) Epitope Mapping of Bovine Serum Albumin Using Monoclonal Antibodies with a Photoreactive Cross-linker. *J. Biochem. 115*, 1119−1127.
(39) Adamczyk, M., Fishpaugh, J., Harrington, C., Hartter, D., Johnson, D., and Vanderbilt, A. (1993) Immunoassay Reagents for Psychoactive Drugs. I. The Method for the Development of Antibodies Specific to Amitriptyline and Nortriptyline. *J. Immunol. Methods 162*, 47−58.
(40) Adamczyk, M., Grote, J., Douglas, J., Dubler, R., and Harrington, C. (1997) Synthesis of Conjugates for a Barbiturate Screening Assay. *Bioconjugate Chem. 8*, 281−288.
(41) Schoneberg, T., Erhard, M. H., Kellner, J., Kuhn, S. O., and Losch, U. (1995) The Specificity and Affinity of Polyclonal Antibodies Raised in Rabbits Against a Hapten Conjugated to a Rabbit Serum Albumin. *J. Immunoassay 16* (1), 17−35.

BC9800320