# Synthesis and Properties of 3-(2-Hydroxyethyl)-3-*n*-pentyldiazirine, a Photoactivable General Anesthetic†

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To overcome the difficulties of locating the molecular sites of general anesthetic action, we synthesized a novel photoactivable general anesthetic, 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (3-diazirinyloctanol), which anesthetized tadpoles with an $ED_{50}$ of 160 $\mu$M. Subanesthetic concentrations of 3-diazirinyloctanol enhanced GABA-induced currents in $GABA_A$ receptors, an effect that has been implicated in general anesthetic action. It also enhanced [$^3$H]muscimol binding to this receptor. In muscle nicotinic acetylcholine receptors (nAcChoR), it inhibited the response to acetylcholine with an $IC_{50}$ of 33 $\mu$M. 3-Diazirinyloctanol's pharmacological actions were comparable to those of octanol. 3-(2-Hydroxyethyl)-3-[4,5-$^3H_2$]-*n*-pentyldiazirine photoincorporated into *Torpedo* nAcChoR-rich membranes mainly in the α subunit with 70% being in a proteolytic fragment containing the M4 transmembrane segment. Agonist enhanced the photolabeling 10-fold in a fragment containing the M1, M2, and M3 transmembrane segments. Thus, 3-diazirinyloctanol is a novel general anesthetic that acts on, and can be photoincorporated into, postsynaptic receptors.

## Introduction

To improve the pharmacological specificity of general anesthetics requires a knowledge of the molecular structure of their sites of action. Currently, there is a consensus that the ligand-gated ion channel superfamily, which includes the $GABA_A$, glycine, nicotinic acetylcholine (nAcChoR), and $5HT_3$ receptors, provides the most plausible targets for general anesthetics.[1] The difficulties of applying X-ray crystallography and NMR to these large membrane proteins means there is no direct evidence for anesthetic binding sites, although such sites do exist on soluble proteins.[2] Furthermore, binding studies encounter severe difficulties with nondisplaceable binding, and the only success remains the case of radiolabeled barbiturates binding to the high-specific-activity acetylcholine receptor from *Torpedo* electroplax.[3]

Indirect evidence for general anesthetic sites on ligand-gated ion channels comes from the allosteric regulation of other ligand binding sites, site-directed mutagenesis,[4−9] and kinetic analysis of the interactions between two inhibitors.[10,11] In the case of the nAcChoR, such studies are consistent with agents such as octanol inhibiting the open channel at a site, probably within the region lined by the second transmembrane helices (M2).[6,11,12] On the other hand, in the $GABA_A$ receptor general anesthetics shift the agonist concentration−response curves to higher apparent affinity,[13] and the case for an anesthetic site rests largely on mutagenesis studies,[7] where clear-cut interpretation is problematic,[14,15] and the stereoselectivity of some intravenous agents.[16]

The difficulty of demonstrating the existence of functional binding sites pales when compared to that of locating such sites. The power of site-directed mutagenesis is blunted by the highly allosteric nature of this superfamily of receptors.[8,9] For example, structural perturbations at the level of nearly all the main structural features of the acetylcholine receptor are known to affect gating. These include the agonist binding domain, the first, second, and fourth transmembrane helices, and the cytoplasmic loop.[17−19]

A complementary technique, photoaffinity labeling, has been successfully employed to identify the amino acids that form the local anesthetic sites on the nAcChoR and the benzodiazepine sites on the $GABA_A$ receptor.[4,20,21] Such an approach has been pioneered for general anesthetics using [$^{14}$C]halothane.[22] However, this agent has low radioactive specific activity, has poorly understood photochemistry, and must be photoactivated at ~250 nm, a wavelength likely to interact with the target protein. Aromatic diazirines have very good photochemical properties and have been widely applied,[23,24] but none have general anesthetic properties. Recent successes with small alkyldiazirines,[25] which are

† Abbreviations: 3-diazirinyloctanol, 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine; [$^3$H]-3-diazirinyloctanol, 3-(2-hydroxyethyl)-3-[4,5-$^3H_2$]-*n*-pentyldiazirine; nAcChoR, nicotinic acetylcholine receptor; LLR, loss of righting reflexes; octanol, *n*-octan-1-ol; TID, 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine; $GABA_A$, $\gamma$-aminobutyric acid type A.

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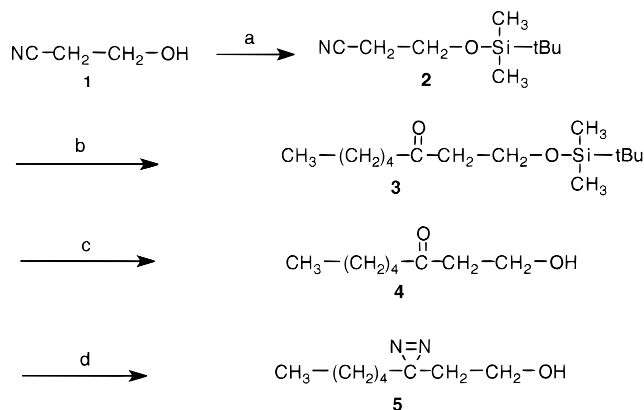
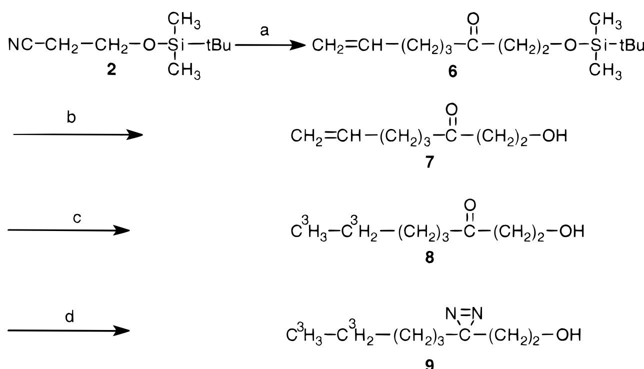
**Scheme 1**[a]




[a] Reagents: (a) $tBuMe_2SiCl$; (b) $CH_3$-$(CH_2)_4$-MgBr; (c) dil HCl; (d) (i) liq $NH_3$, $NH_2$-O-$SO_2OH$, (ii) $I_2$, $Et_3N$.

**Scheme 2**[a]




[a] Reagents: (a) $CH_2$=CH-$(CH_2)_3$-MgBr; (b) dil HCl; (c) tritium, Pd−carbon; (d) (i) liq $NH_3$, $NH_2$-O-$SO_2OH$, (ii) $I_2$, $Et_3N$.

closer in struture to conventional anesthetics, stimulated us to synthesize unlabeled and tritiated 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (3-diazirinyloctanol). We show here that 3-diazirinyloctanol behaves like a reversible general anesthetic in three widely accepted models (tadpoles, the $GABA_A$ and nicotinic receptors) and that it may be photoincorporated into acetylcholine receptors in a state-dependent manner.

## Chemistry

Scheme 1 shows the strategy for the synthesis of 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine. It involved protection of the hydroxyl function of the starting material, 3-hydroxypropionitrile, with the acid-labile *tert*-butyldimethylsilyl group and Grignard reaction of the protected nitrile with pentylmagnesium bromide followed by deprotection of the resulting ketone. The hydroxy ketone was then treated with hydroxylamine-*O*-sulfonic acid in liquid ammonia to give the corresponding diaziridine derivative which was subsequently oxidized to the diazirine.

The synthesis of tritiated 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (Scheme 2) involved Grignard reaction of *tert*-butyldimethylsilyl-protected hydroxypropionitrile with pentenylmagnesium bromide to give the protected unsaturated hydroxy ketone. Deprotection followed by catalytic tritiation with carrier-free tritium gave 1-hydroxy-[7,8-$^3H_2$]-3-octanone. Conversion of the labeled hydroxyoctanone to 3-(2-hydroxyethyl)-3-[4,5-$^3H_2$]-*n*-

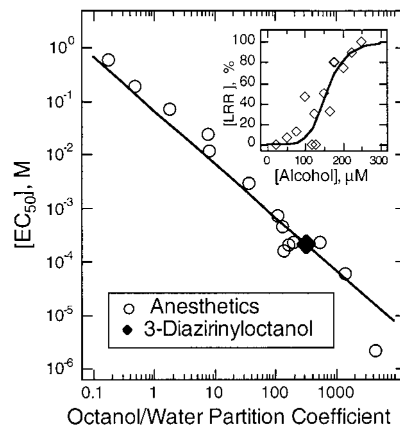



**Figure 1.** 3-Diazirinyloctanol is a general anesthetic. Inset: 3-Diazirinyloctanol caused loss of righting reflexes in tadpoles. Each point represents the mean response of 5−15 animals. Main figure: 3-Diazirinyloctanol (filled diamond) obeys the Meyer−Overton rule relating $EC_{50}$ to octanol/water partition coefficient. The circles are data taken from Firestone[39] and include volatile anesthetics, alcohols, barbiturates, and propofol.[40] In order of decreasing potency the agents plotted are propofol, octanol, pentobarbital, methoxyflurane, heptanol, halothane, amobarbital, hexanol, pentanol, butanol, diethyl ether, propanol, ethanol, and methanol. The theoretical line was fitted by linear least squares with a slope of −1.

pentyldiazirine was then accomplished as with the unlabeled hydroxy ketone.

## Results

**Physical Properties.** 3-Diazirinyloctanol could be stored in ethanol (~500 mM) in amber reacti-vials with Teflon-lined septums at −80 °C for at least 3 months. Aqueous solutions were stable in the dark overnight and for up to 5 h under normal laboratory lights. 3-Diazirinyloctanol had a major absorption peak at 350 nm and a smaller one at 366 nm, characteristic of the diazirine group. Depending on the degree of exposure, 366-nm light caused decomposition over minutes to tens of minutes. 3-Diazirinyloctanol was absorbed completely from aqueous solution by polypropylene tubes. The solubility of 3-diazirinyloctanol in water was found to be 11 mM at room temperature, and the octanol/water partition coefficient was 330 ± 25 ($n$ = 3; errors are given as standard deviations throughout).

**General Anesthetic Potency.** A total of 125 tadpoles were exposed in groups of 5 to concentrations of 3-diazirinyloctanol ranging from 50 to 250 $\mu$M. The steady-state level of the loss of righting reflexes (LRR) was established between 10 and 15 min. The response was routinely measured after 20 min, and all tadpoles were then transferred to oxygenated fresh water and allowed to recover from anesthesia. All animals recovered from exposure to concentrations up to 250 $\mu$M, but at 400 $\mu$M many did not recover. The data were fitted to a logistic curve to give an $EC_{50}$ of 160 ± 8 $\mu$M and a slope of 6.1 ± 1.8 (Figure 1). Similarly 50 tadpoles were exposed to concentrations of octanol ranging from 15 to 120 $\mu$M, yielding an $EC_{50}$ of 60 ± 6.3 $\mu$M and a slope of 2.3 ± 0.59, similar to a value previously reported.[26]

**Actions on the $GABA_A$ Receptor Physiology.** In human $GABA_A$ receptors comprised of $\alpha_1$, $\beta_2$, and $\gamma_{2L}$ subunit isoforms expressed in oocytes, 3-diazirinyloctanol strongly and reversibly potentiated GABA-elicited

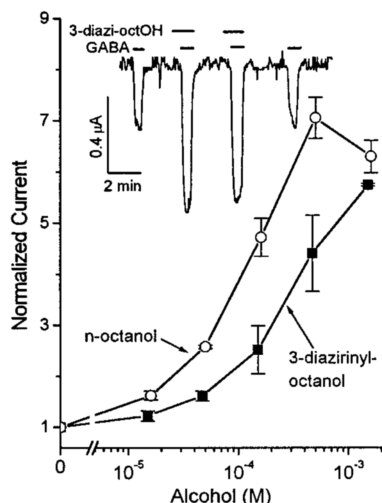



**Figure 2.** 3-Diazirinyloctanol potentiates $GABA_A$ receptor function. Inset: The GABA-induced current recorded from a whole oocyte injected with $GABA_A$ receptor $\alpha_1$, $\beta_2$, and $\gamma_{2L}$ subunit cRNAs demonstrates reversible potentiation by 100 $\mu$M 3-diazirinyloctanol (3-diazi-octOH). The potentiation of GABA-activated currents by 3-diazirinyloctanol and octanol is concentration-dependent. Currents were elicited with 10 $\mu$M GABA, and the drug was presented in both control and GABA solutions as shown by the bars in the inset. Data points represent the average (±SD) of measurements from at least 3 separate oocytes, normalized to currents elicited with 10 $\mu$M GABA alone.

currents when it was co-applied with the agonist (see inset Figure 2). Using a GABA concentration (10 $\mu$M) which gave 5% of the maximum current, subanesthetic concentrations of 3-diazirinyloctanol caused a significant 1.6-fold enhancement at 47 $\mu$M ($p < 0.01$), which increased without the effect saturating up to the highest concentration examined—1.5 mM where currents were increased 5.7-fold.

Octanol was more potent than 3-diazirinyloctanol but equally efficacious, causing equivalent current enhancement at about 3−4-fold lower concentrations. Octanol potentiation showed a peak near 500 $\mu$M, and higher concentrations did not cause further current enhancement. Neither alcohol stimulated currents in the absence of agonists.

**Allosteric Regulation of the $GABA_A$ Receptor.** A typical experiment with 2 mg/mL bovine cortex membranes and 7 nM [$^3$H]muscimol gave total binding of 2200 dpm with a background of 530 dpm. 3-Diazirinyloctanol and octanol both caused a similar concentration-dependent enhancement of [$^3$H]muscimol binding to $GABA_A$ receptors reaching 150−160% of control at 1 mM, the highest concentration examined (Figure 3).

**Actions on the Nicotinic Receptors.** When 3-diazirinyloctanol was co-applied with acetylcholine to membrane patches excised from oocytes expressing recombinant mouse muscle receptors, it inhibited currents (see inset of Figure 4) with an $IC_{50}$ of 33 ± 2.6 $\mu$M and a Hill coefficient of 1.0 ± 0.05. This inhibition was fully reversible.

**Photoincorporation of [$^3$H]-3-Diazirinyloctanol into nAcChoR-Rich Membranes.** Initial experiments were designed to characterize the general pattern of photoincorporation of [$^3$H]-3-diazirinyloctanol and to test the sensitivity of photoincorporation to various ligands. Membranes were equilibrated with 1 $\mu$M [$^3$H]-3-diazirinyloctanol in the presence and absence of 2 mM carbamylcholine. After irradiation for 10 min, the pattern of incorporation was assessed by SDS−PAGE followed by fluorography. The main site of incorporation within the nAcChoR was in the α-subunit (Figure 5, lanes 2−3), and that incorporation was not seen in the absence of UV irradiation (Figure 5, lane 4). Incorporation into the α-subunit was dependent on the conformational state of the receptor, as the presence of agonist (Figure 5, lane 3) resulted in enhanced incorporation into the α-subunit but not in non-nAcChoR polypeptides. In addition to the α-subunit, there was UV-dependent incorporation into a 34-kDa polypeptide, identified as a mitochondrial chloride channel.[27] There was small and variable amount of incorporation of [$^3$H]-3-diazirinyloc-

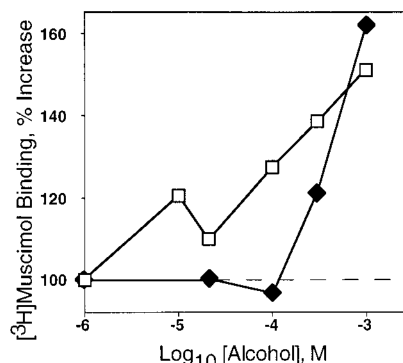



**Figure 3.** 3-Diazirinyloctanol potentiates the specific binding of a $GABA_A$ receptor agonist, [$^3$H]muscimol. Open squares are octanol, and closed diamonds are 3-diazirinyloctanol. Bovine cerebral cortex membranes were incubated with ligands for 30 min at 21 °C before being filtered and washed as described in The Experimental Section. Points are the mean of triplicates whose average standard deviation was 10%.

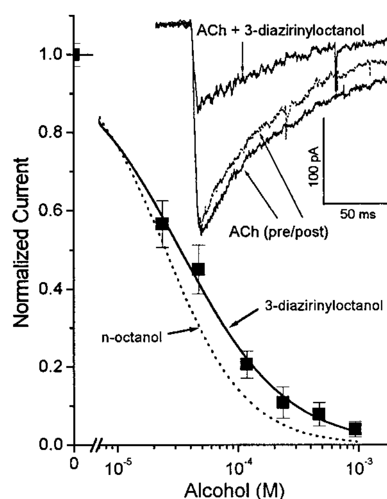



**Figure 4.** 3-Diazirinyloctanol inhibits nicotinic acetylcholine receptor function. Inset: 50 $\mu$M reversibly inhibits macroscopic patch currents elicited by a saturating AcCho concentration (200 $\mu$M). Currents were recorded from an excised outside-out membrane patch from an oocyte injected with mouse nicotinic receptor $\alpha_1$, $\beta_1$, $\gamma_1$, and $\delta_1$ subunit cRNAs. Main figure: AcCho-activated current inhibition by 3-diazirinyloctanol is concentration-dependent. Data points represent the average (±SD) of measurements from at least 3 separate patches. A logistic function was fitted to the data points (solid line) using nonlinear least squares: $IC_{50} = 33 \pm 2.6$ $\mu$M; $n_H = 1.0 \pm 0.05$. An octanol concentration−response relationship (dotted line) from ref 6 is shown for comparison; it has an $IC_{50}$ of 23 $\mu$M and a Hill coefficient of 1.3.

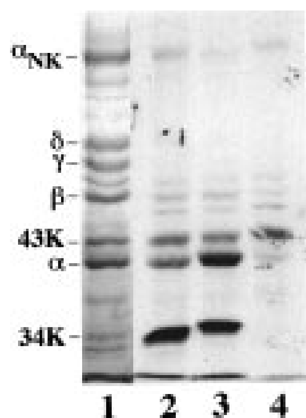



**Figure 5.** [$^3$H]-3-Diazirinyloctanol photoincorporates into *Torpedo* nAcChoR. nAcChoR-rich membranes were photolabeled with [$^3$H]-3-diazirinyloctanol as described in the Experimental Section. Polypeptides were resolved by SDS−PAGE, visualized by Coomassie Blue stain (lane 1), and processed for fluorography (15-day exposure, lanes 2−4). Membrane suspensions equilibrated with 1 μM [$^3$H]-3-diazirinyloctanol in the absence (lane 2) and presence (lane 3) of 2 mM carbamylcholine were irradiated for 10 min. Lane 4: nonirradiated aliquot of membranes equilibrated with [$^3$H]-3-diazirinyloctanol in the presence of 2 mM carbamylcholine. Indicated on the left are the mobilities of the nAcChoR subunits (α, β, γ, δ), rapsyn (43K), the α-subunit of the $Na^+$/$K^+$ATPase ($\alpha_{NK}$), and mitochondrial VDAC (34K).

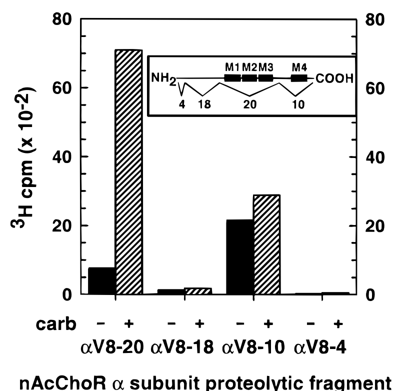



**Figure 6.** Mapping of [$^3$H]-3-diazirinyloctanol photoincorporation into nAcChoR α-subunit by use of *S. aureus* V8 protease. nAcChoR-rich membranes were photolabeled with [$^3$H]-3-diazirinyloctanol in the absence (solid bars) or presence (hatched bars) of 2 mM carbamylcholine. Polypeptides were resolved by SDS−PAGE on an 8% gel, and the α-subunit was excised and transferred to the wells of a 15% mapping gel for digestion with 5 μg of V8 protease as described in the Experimental Section. The mapping gel was stained with Coomassie blue to identify the four subunit fragments produced by V8 protease, and tritium incorporation in the fragments was determined by liquid scintillation counting. Inset: Schematic showing the approximate distribution within the α-subunit primary structure of the fragments produced by cleavage by *S. aureus* V8 protease.

tanol that did not depend on photolysis, primarily within rapsyn (43K), a cysteine-rich, nAcChoR-associated, peripheral protein.[28]

To further localize the sites of incorporation of [$^3$H]-3-diazirinyloctanol, labeled α-subunit was digested with *S. aureus* V8 protease to generate four large, nonoverlapping fragments resolvable by SDS−PAGE. A 20-kDa peptide (αV8−20), begins at Ser-173 and contains the first three membrane spanning regions, M1, M2, and M3.[29] A 10-kDa peptide (αV8−10) contains the fourth membrane spanning region, M4, and begins at Asn-339.[30] A 18-kDa peptide (αV8−18) and a 4-kDa peptide (αV8−4) begin at Val-46 and Ser-1, respectively. Scintillation counting of these four proteolytic fragments showed the main site of photoincorporation in the absence of agonist to be the αV8−10 fragment, which contained 70% of tritium (Figure 6). Agonist increased the labeling of the αV8−20 fragment 9-fold, while the labeling of αV8−10 was unchanged, resulting in 70% tritium incorporation in αV8−20 and 28% in αV8−10.

## Discussion

**General Anesthetic Properties.** The tadpole is a traditional animal for measuring in vivo general anesthetic potency when limited quantities of material are available. 3-Diazirinyloctanol behaved as a typical general anesthetic, having a potency between that of heptanol and octanol. General anesthetic potency has long been known to correlate well with lipophilicity as represented by the olive oil or octanol to water partition coefficients. This, the Meyer−Overton rule, is illustrated for 14 general anesthetics ranging from volatile agents to barbiturates in Figure 1. It can be seen that the loss of potency upon substitution of the diazirine group into octanol is satisfactorily predicted by 3-diazirinyloctanol's measured octanol/water partition.

**Actions on the $GABA_A$ Receptor.** A strong case can be made that general anesthetic−$GABA_A$ receptor interactions play a primary role in the induction of the state of general anesthesia.[13,31,32] At concentrations in the clinical range, alcohols, like most other general anesthetics, enhance inhibitory currents elicited by concentrations of GABA below those causing maximum stimulation.[33] At equipotent concentrations in tadpoles, octanol (50 μM) and 3-diazirinyloctanol (150 μM) enhanced currents elicited by 10 μM GABA equally by 2.6 ± 0.04 and 2.5 ± 0.47 times, respectively. Furthermore, the allosteric actions of 3-diazirinyloctanol resemble those of other general anesthetics, all of which enhance muscimol binding independent of brain region.[32]

**Actions on the nAcChoR.** The muscle subtype acetylcholine receptor remains an important model for exploring the molecular mechanisms by which general anesthetics act on ligand-gated ion channels.[6,10] Furthermore, its superior abundance in *Torpedo* electroplaques has allowed the direct contact points of several ligands to be obtained by photolabeling (reviewed in ref 20).

A strong but indirect case that octanol inhibits the open state of the nAcChoR by binding to a site in the channel lumen that is distinct from the local anesthetic site has been built on kinetic analysis[6] and mutagenesis studies[11] which by their very nature are subject to alternative interpretations. Because 3-diazirinyloctanol inhibits the open channel state of the muscle type nAcChoR with a Hill coefficient of 1, just like octanol,[6] it should be useful for resolving these ambiguities by establishing the contact points of the agent with the inhibitory site on nAcChoRs from *Torpedo* electroplax.

**Photoincorporation into the nAcChoR.** [$^3$H]-3-Diazirinyloctanol was successfully and selectively photoincorporated into acetylcholine receptor-rich membranes from *T. californica*. Incorporation occurs preferen-

tially within the α-subunits and is increased 3-fold on conversion of the nAcChoR into the desensitized state by addition of carbamylcholine. Because alcohols can stabilize the desensitized state in the absence of agonist,[34,35] we expect that at least part of that enhanced incorporation results because 3-diazirinyloctanol is bound with higher affinity by desensitized nAcChoRs. Agonist-sensitive photoincorporation (~3000 cpm) into the excised α-subunit indicates that ~2% of α-subunits contain tritium. To reduce nonspecific incorporation, the concentration used for the labeling study (1 μM) was much less than the concentration required for channel inhibition ($IC_{50}$ ~50 μM, Figure 4) and very much less than that expected to cause desensitization (~0.5 mM).[35] Once the precise points of photoincorporation have been defined, detailed study of their concentration dependencies and pharmacologies will be required to establish which sites are of functional significance.

The strong α-subunit selectivity of [$^3$H]-3-diazirinyloctanol contrasts with other pharmacologically distinct drugs that photoincorporate within the M2 helix that lines the lumen of the ion channel of the nAcChoR. The aromatic amines [$^3$H]chlorpromazine and [$^3$H]triphenylmethylphosphonium both incorporate with similar efficiency into M2 segments in each nAcChoR subunit in the desensitized state, while [$^{125}$I]-3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (TID) is photoincorporated with similar efficiency into each nAcChoR subunit in the resting state (reviewed in ref 20). Thus, the selective labeling of the α-subunit by [$^3$H]-3-diazirinyloctanol may indicate a novel binding domain.

The enhanced photoincorporation of [$^3$H]-3-diazirinyloctanol in the desensitized state contrasts strongly with the state dependence of the aromatic diazirines, [$^{125}$I]-TID[30] and [$^3$H]diazofluorene,[27] both of which incorporate efficiently only in the resting state even though they bind reversibly within the ion channel with similar affinities in both states.

Taken together, the pattern of subunit selectivity and state dependence of photoincorporation into the nAcChoR suggests that 3-diazirinyloctanol interacts with a novel binding site. Further experiments should provide a clearer localization of this long-chain alcohol binding site on the nAcChoR.

## Conclusions

We have synthesized a novel general anesthetic, which, when activated by nondamaging UV wavelengths, photoincorporates into a membrane protein with good yield. Pharmacological studies show that the photoprobe exhibits many of the properties expected of a true general anesthetic. It thus provides a promising tool for locating at the amino acid residue level the sites of action of general anesthetics on their target proteins.

## Experimental Section

**Materials.** Octanol, *tert*-butyldimethylsilyl chloride, 3-hydroxypropionitrile, hydroxylamine-*O*-sulfonic acid, and pentylmagnesium bromide were obtained from Aldrich (Milwaukee, WI). Solutions of 3-diazirinyloctanol in ethanol (460 mM) were stored in the dark at −80 °C until needed.

**Preparation of 3-(*tert*-Butyldimethylsilyloxy)propionitrile (2).** A mixture of *tert*-butyldimethylsilyl chloride (**1**) (200 mL, 1 M solution in anhydrous tetrahydrofuran), 3-hydroxypropionitrile (13.5 g, 0.19 mol), and imidazole (31.25 g, 0.46 mol) was stirred for 15 h at 40 °C under argon. The reaction mixture was twice extracted with 100-mL portions of water and the organic layer dried over anhydrous $MgSO_4$. After removal of the solvent by rotary evaporation, the crude product was purified on a column of silica gel using hexane: dichloromethane (1:1 v/v) as an eluant to give 34.5 g (98% yield) of the colorless liquid product (**2**). TLC: $R_f$ 0.56 (dichloromethane). Anal. ($C_9H_{19}NOSi$) C, H, N.

**Preparation of 1-(*tert*-Butyldimethylsilyloxy)octan-3-one (3).** Pentylmagnesium bromide (30 mL, 2 M solution in anhydrous diethyl ether) was slowly added under argon to a stirred solution of the cyano compound **2** (24.7 g, 0.133 mol) at 0 °C. The solution was then stirred at room temperature overnight. After addition of ice (200 g), the reaction mixture was acidified under vigorous stirring with concentrated HCl to pH 5 and stirred for 2 h. The ether layer was separated from the mixture, the aqueous layer re-extracted with ether, and the combined ether layer dried over anhydrous $MgSO_4$. After removal of the ether by rotary evaporation, the crude product (**3**) was purified on a silica gel column equilibrated with hexane to give 18.8 g (54% yield) of an oily product. TLC: $R_f$ 0.39 (hexane:ether, 9:1). Mass spectrum: $m/z = 259$ $(M^+ H)^+$ was consistent with 1-(*tert*-butyldimethylsilyloxy)octan-3-one (**3**). Anal. ($C_{14}H_{30}OSi$) C, H, N.

**Preparation of 1-Hydroxyoctan-3-one (4).** The keto derivative **3** (5.2 g, 0.02 mol) was dissolved in 25 mL of concentrated HCl−methanol mixture (1:10 v/v) and incubated at room temperature for 90 min. The mixture was diluted with 150 mL of ether and extracted twice with 100-mL portions of 5% $Na_2CO_3$. After drying over anhydrous $MgSO_4$, the ether layer was evaporated and the oily residue purified on a silica gel column equilibrated with $CH_2Cl_2$:hexane (3:1 v/v). Elution with $CH_2Cl_2$:ether (9:1 v/v) gave 1.29 g (45% yield) of an oily product. TLC: $R_f$ 0.27 (dichloromethane:ether, 9:1). Mass spectrum: $m/z = 162$ $(M^+ NH_4)^+$ was consistent with 1-hydroxyoctan-3-one (**4**). Anal. ($C_8H_{16}O_2$) C, H, N.

**Preparation of 3-(2-Hydroxyethyl)-3-*n*-pentyldiazirine (5).** Anhydrous ammonia (10 mL) was condensed into a round-bottomed flask containing 1-hydroxyoctan-3-one (**4**) (1.25 g, 8.7 mmol) at dry ice temperature. The mixture was stirred at −35−40 °C for 5 h. The solution was cooled with dry ice, and a solution of hydroxylamine-*O*-sulfonic acid (1.13 g, 10 mmol) in anhydrous methanol (6 mL) was added over a period of 30 min. The dry ice bath was removed, and the mixture was refluxed with stirring at −35 °C for 1 h. The ammonia was then allowed to evaporate overnight. The resulting slurry was filtered and the filter cake washed with several portions of methanol. The combined solution was rotary evaporated. The residue of the diaziridine derivative was dissolved in dichloromethane (5 mL) and treated with triethylamine (1.5 mL). A solution of iodine (1.6 g, 12.5 mmol) in dichloromethane (10 mL) was slowly added with stirring until the appearance of a persistent orange-brown coloration. The mixture was chromatographed on a column of silica gel equilibrated with dichloromethane−hexane (4:1 v/v). After successive washing of the column with this solvent and then with dichloromethane, the major fraction with absorbance at 350 nm was eluted with dichloromethane containing 5% ether to give 318 mg (27% yield) of 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (**5**). TLC: $R_f$ 0.54 (dichloromethane:ether, 9:1). UV spectrum: $\lambda_{max}$ (methanol) 350 nm, $\epsilon = 59$ $M^{-1}$ $cm^{-1}$, both characteristic of the diazirine group.[36] Mass spectrum: $m/z = 174$ $(M^+ NH_4)^+$ was consistent with 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (**5**). Anal. ($C_8H_{16}N_2O$) C: calcd, 61.51; found, 60.86. H: calcd, 10.32; found, 10.38. N: calcd, 17.93; found, 17.32. The product was stored at −80 °C in dark.

**Preparation of 1-(*tert*-Butyldimethylsilyloxy)-7-octen-3-one (6).** Bromo-1-pentene (18.8 g, 126 mmol) in anhydrous ether (100 mL) was stirred with magnesium turnings (2.8 g, 115 mmol) under argon for 1 h at room temperature. The mixture was cooled to −10 °C, and a solution of 3-(*tert*-butyldimethylsilyloxy)propionitrile (**2**) (12.4 g, 66.8 mmol) in anhydrous ether (10 mL) was slowly added. The mixture was stirred under argon overnight at room temperature. Ice (200

g) was then added to the reaction mixture and the suspension acidified under vigorous stirring with concentrated HCl to pH 5. After 2 h, the ether layer was separated from the mixture, the aqueous layer re-extracted with ether, and the combined ether layer dried over anhydrous $MgSO_4$. After removal of the ether by rotary evaporation, the crude product was purified on a silica gel column as described for the purification of 1-(*tert*-butyldimethylsilyloxy)octan-3-one (**3**) to yield 6.8 g of the unsaturated, protected hydroxy ketone (**6**). Anal. ($C_{14}H_{28}OSi$) C, H, N.

**Preparation of 1-Hydroxy-7-octen-3-one (7).** 1-(*tert*-Butyldimethylsilyloxy)-7-octen-3-one (**6**) (5.2 g, 0.02 mol) was deprotected to 3-oxo-7-octen-1-ol (**7**) and purified as described for the saturated analogue (**4**).

**Preparation of 1-Hydroxy-[7,8-$^3H_2$]-octan-3-one (8).** Reductive tritiation of 1-hydroxy-7-octen-3-one (**7**) was performed by New England Nuclear Corp. using carrier-free tritium gas and palladium charcoal to give 1-hydroxy-[7,8-$^3H_2$]-octan-3-one (**8**).

**Preparation of 3-(2-Hydroxyethyl)-3-[4,5-$^3H_2$]-*n*-pentyldiazirine (9).** This operation involving a large amount of volatile radioactive material was carried out in a Plexiglas box which was exhausted through filters containing silica gel and activated carbon. Unlabeled 1-hydroxyoctan-3-one (**4**) (1 mg, 7 mmol) in 10 $\mu$L of methanol was mixed with [$^3$H]-1-hydroxyoctan-3-one (**8**) (200 mCi) in a microhydrolysis vacuum tube (Kontes, Vineland, NJ). The tube was cooled in an acetone−dry ice bath, and anhydrous ammonia (0.4 mL) was added to the tube. The tube was sealed, and the reaction mixture stirred at −35−40 °C for 5 h. The tube was then cooled to dry ice temperature, and a solution of hydroxylamine-*O*-sulfonic acid (1.25 mg, 11 mmol) in anhydrous methanol (25 $\mu$L) was added over a period of 20 min. The mixture was stirred in the sealed tube for 1 h at −40 °C and then at room temperature overnight. The tube was unsealed, and the ammonia was allowed to evaporate for 2 h. The residue of the diaziridine derivative was dissolved in dichloromethane (0.2 mL), treated with triethylamine (10 $\mu$L), and cooled in an ice bath. A solution of iodine (2.54 mg) in dichloromethane (0.1 mL) was slowly added until a reddish brown color persisted. The mixture was diluted with 0.3 mL of hexane and the mixture chromatographed on a column of silica gel (7 mL) equilibrated with dichloromethane−hexane (3.72:1.25 v/v). After successive washing of the column with the equilibration buffer and dichloromethane, elution with dichloromethane: ether (19:1) yielded 25 mCi of 3-(2-hydroxyethyl)-3-[4,5-$^3H_2$]-*n*-pentyldiazirine (**9**). The product was chemically and radiochemically pure by TLC and HPLC and eluted congruently with unlabeled 3-(2-hydroxyethyl)-3-*n*-pentyldiazirine (**5**). The specific activity was determined by HPLC within 10% to be 11 Ci/mmol by comparison of the labeled compound with a calibration curve for the pure unlabeled compound obtained at 360 nm.

**General Anesthetic Potency.** With institutional approval, general anesthetic potency was assessed in prelimb bud *Rana pipiens* tadpoles, approximately 2 cm in length (Carolina Biological Supply Co., Burlington, NC). Groups of 5 tadpoles were placed in covered 100-mL beakers in oxygenated aqueous solutions of the alcohol under low levels of ambient light. 3-Diazirinyloctanol was added from stock solutions in ethanol. The final concentration of ethanol did not exceed 5 mM. Previous work has shown that 25 mM ethanol did not affect the response to octanol.[26] Anesthesia was defined as the LRR. Tadpoles were tipped manually with a flame polished pipet, and failure to right after 5 s was defined as LRR. The concentration−response curves were analyzed by fitting to a logistic function.

**Solubility Properties.** The solubility in water was determined by shaking with excess 3-diazirinyloctanol (30 mM) for 3 h, centrifuging at 10000*g* for 3 min, and determining the concentration at 350 nm. The octanol/water partition coefficient was determined by shaking a two-phase mixture in an amber vial and allowing it to settle for 2 h before withdrawing aliquots from each phase for analysis on an HPLC (reverse-phase C-18 column (Vydac, Hesperia, CA); water−acetonitrile gradient at 1 mL/min; detection at 214 nm).

**Electrophysiology.** Recombinant human $GABA_A$ receptors ($\alpha_1$, $\beta_2$, and $\gamma_{2L}$ subunits) and mouse muscle nAcChoR ($\alpha_1$, $\beta_1$, $\gamma_1$, and $\delta_1$ subunits) were expressed in *Xenopus* oocytes for electrophysiology.[6] $GABA_A$ receptor subunit cDNAs were a gift of Dr. Paul Whiting (Merck Neuroscience, UK) and nicotinic receptor cDNAs were a gift of Dr. James McLaughlin (Tufts Medical School, Boston, MA). Capped messenger RNAs were in vitro transcribed from linearized cDNAs (mMessage mMachine; Ambion, Austin, TX). Appropriate mRNA mixtures were microinjected into oocytes (25−50 ng) and incubated in ND-96 solution (in mM: 96 NaCl, 2 KCl, 0.8 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, pH 7.6) containing 100 U/mL penicillin and 100 g/mL streptomycin for 48−72 h.

$GABA_A$ receptor chloride currents were recorded in whole oocyte studies at room temperature (20−22 °C). Oocytes were placed in a flow cell (150 $\mu$L), impaled with 3 M KCl-filled glass microelectrodes (0.5−2.0 MΩ bath resistance), and clamped at −50 mV. ND-96 superfusate solutions were controlled by an upstream valve. GABA was superfused for sufficient time to ensure that a steady-state or peak current was achieved before returning to control superfusate and a recovery period of at least 2 min was allowed between GABA exposures. In experiments where *n*-octanol or 3-diazirinyloctanol was added to superfusates, control currents without the alcohol were recorded at every second or third trial.

Nicotinic receptor currents were recorded from oocyte membrane patches at room temperature. Details of the rapid superfusate application and electrophysiologic recording methods have been reported.[6] Patches were exposed to ACh solutions at 10 × $EC_{50}$ (200 $\mu$M) in K-100 (in mM: 97 KCl, 1 $MgCl_2$, 0.2 EGTA, 5 K-HEPES, pH 7.5) in a series of 8−16 pulses lasting 300 ms with interpulse periods of 10 s in control solution. *n*-Octanol or 3-diazirinyloctanol was added to both control and ACh superfusate solutions, and control currents without the compounds were recorded at every other trial.

*n*-Octanol and 3-diazirinyloctanol solutions were prepared from ethanol stocks immediately prior to use in electrophysiology experiments. Since ethanol is a known potentiator of $GABA_A$ receptor currents, we measured the potentiation caused by the maximum ethanol concentration (0.3% v/v = 65 mM). This ethanol concentration produced less than 5% potentiation of $GABA_A$ receptor currents and no effect on nicotinic receptor currents.

**Data Analysis.** For patch currents, the data from each series of 8−16 agonist/drug exposures were averaged to produce an ensemble average current. For both whole oocyte and patch currents, baseline leak currents were subtracted and peak agonist-activated currents were measured directly from traces. Control currents (agonist alone) were checked before and after experiments where patches were exposed to alcohols. Data were not analyzed if the pre- and post-control peak currents differed by more than 10%. Currents from experiments where alcohol was present were normalized to the average of pre- and post-control currents. Concentration−response data were fitted with logistic functions by the method of nonlinear least squares (Origin; Microcal Inc., Northampton, MA).

**Binding Studies on the $GABA_A$ Receptor.** Bovine cerebral cortex was obtained fresh from a local slaughter-house, dissected, and stored at −70 °C for up to 1 year. The tissue was thawed and homogenized at 0−4 °C, and a low-speed pellet was removed. The crude mitochondrial, synaptosomal, and microsomal membranes were collected by high-speed centrifugation, resuspended in water for osmotic shock, recentrifuged, and washed three times in assay buffer. The homogenate was suspended at ∼1 mg/mL protein in 0.1 M KCl, 20 mM K-phosphate buffer, pH 7.5, for assay; 400-$\mu$L aliquots in at least triplicate were incubated in capped tubes for 30 min at 21 °C with 5−7 nM [$^3$H]muscimol (20 Ci/mmol; NEN, Boston, MA) and varying amounts of modulatory drugs, final volume 500 $\mu$L. The concentration of ethanol never exceeded 34 mM, which caused no change in the binding of either ligand.

The nonspecific binding of [$^3$H]muscimol was estimated with 0.1 mM GABA or 10 mM picrotoxin, respectively. Samples were vacuum-filtered (GF/B, Whatman, Hillsboro, OR) with a Brandel cell harvester (Gaithersburg, MD), with 3 × 2-mL rinses with cold buffer. Filters were counted for radioactivity in Cytoscint (ICN, Costa Mesa, CA; efficiency, 50%).

**Photoincorporation of [$^3$H]-3-Diazirinyloctanol into the nAcChoR.** nAcChoR-rich membranes, prepared as described from *T. californica* electric organ,[37] were suspended in *Torpedo* physiological saline (TPS, in mM: 250 NaCl, 5 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 5 sodium phosphate, pH 7.0) at a final concentration of 2 mg/mL; 50-$\mu$L aliquots of membrane suspensions were incubated with [$^3$H]-3-diazirinyloctanol in the absence or presence of ligands, and the samples were irradiated at 4 °C at a distance of 6 cm with a 365-nm lamp (EN-Spectroline). Electrophoresis sample-loading buffer was added to the samples, and they were submitted to SDS−PAGE on 1.0-mm thick 8% polyacrylamide gels with 0.33% bis(acrylamide).[29] Polypeptides were visualized by staining with Coomassie Blue R-250 (0.25% w/v in 45% methanol and 10% acetic acid) and destaining in 25% methanol, 10% acetic acid. The gels were then impregnated with fluor (Amplify, Amersham Pharmacia Biotech) for 20 min with rapid shaking, dried, and exposed at −80 °C for 2 weeks. Liquid scintillation counting was used to quantify the incorporation of radioactivity into individual peptides cut from stained polyacrylamide gels. Gel slices were added to 5 mL of a solubilization cocktail consisting of 10% (v/v) tissue solubilizer (TS-2, RPI), 2.8 g/L 2,5-diphenyloxazole, and 0.28 g/L *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene. Proteolytic digestion of the isolated α-subunit with *S. aureus* V8 protease was performed in gel as described by White.[38] Photolabeling was carried out with 400-$\mu$g aliquots of nAcChoR membranes and 4 $\mu$M [$^3$H]-3-diazirinyloctanol, and the α-subunits were excised from the first 8% gel and transferred to 15% "mapping gel" for digestion with V8 protease.

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