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Affinity Probes for the GABA-Gated Chloride Channel: 5*e*-*tert*-Butyl-2*e*-[4-(substituted-ethynyl)phenyl]-1,3-dithianes with Photoactivatable, Fluorescent, Biotin, Agarose and Protein Substituents

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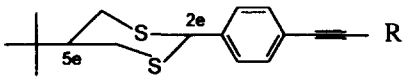
Abstract—Affinity probes for the noncompetitive blocker or picrotoxinin site of the γ-aminobutyric acid (GABA)-gated chloride channel were designed for four types of applications: photoaffinity reagents to covalently label the binding site; fluorescent probes for receptor analysis; biotinylated compounds and agarose/sepharose conjugates for affinity chromatography; ligand–protein/enzyme conjugates for immunoassay. These 5*e*-*tert*-butyl-2*e*-[4-(substituted-ethynyl)phenyl]-1,3-dithianes were optimized by structure–activity studies for potency as inhibitors of ^{3}H ethynylbicycloorthobenzoate binding to bovine brain membranes, measured as the concentration for 50% inhibition (IC_{50}). Preferred compounds are 5*e*-$(CH_3)_3CCH(CH_2S)_2CH$-2*e*-C_6H_4-4-$C{\equiv}CCH_2OCH_2C(O)R$, wherein R confers the following properties and IC_{50} values: R = $SCH_2CH_2SCH_2C(O)C_6H_4$-4-N_3, photoaffinity, 9 nM; R = $NHCH_2CH_2NHC(O)C_6H_2$-2-OH,5-I,4-N_3, photoaffinity, 105 nM; R = SCH_2CH_2S-4-benzofurazan-7-NO_2, fluorescent, 13 nM; R = $SCH_2CH_2SCH_2$-5-fluorescein, fluorescent, 27 nM; R = $NHCH_2CH_2NH[C(O)(CH_2)_5NH]_2$-biotin, affinity chromatography, 190 nM. The most potent photoaffinity ligand (IC_{50} 9 nM) was labeled at 7 Ci mmol^{-1} by reacting the appropriate thiol with ^{3}H 4-azidophenacyl bromide (obtained by alumina-catalyzed tritium exchange of its enolizable hydrogens). The first steps have been taken in using the NCB site for affinity chromatography of the $GABA_A$ receptor in CHAPS-solubilized bovine brain membranes with the dithiane–biotin probe and an avidin–acrylic bead system or with an analogous dithiane–agarose/sepharose column eluting with GABA or dithiane as above (R = OH). A protein conjugate of a related dithiane–monosulfone elicited production of specific antisera in rabbits. These findings illustrate the diversity and utility of new affinity probes prepared in the alkynylphenyldithiane series.

Introduction

The noncompetitive blocker (NCB) site of the γ-aminobutyric acid (GABA)-gated chloride channel is the target for picrotoxinin, several insecticides (e.g. lindane and cyclodienes) and convulsants (bicyclophosphorus esters) and many heterocyclic compounds with high insecticidal activity (2,6,7-trioxabicyclo[2.2.2]octanes and 1,3-dithianes).[1,2] This site is readily examined with trioxabicyclooctane radioligands that act by blocking the chloride channel.[2–4] Trioxabicyclooctanes and dithianes have also been modified to obtain photoactivatable irreversible probes,[5–8] although these candidates have not been radiolabeled for use as photoaffinity ligands. Cyclodiene–protein conjugates have been used to produce antibodies for immunoassay.[9,10] Affinity probes based on the NCB site have not been reported for fluorescence analysis and affinity chromatography of the GABA receptor.

Two steps have been taken to develop 1,3-dithianes as affinity probes for the NCB site of the GABA-gated chloride channel. The first was the recognition that 5*e*-*tert*-butyl-2*e*-[(4-substituted-ethynyl)phenyl]-1,3-dithianes were an appropriate choice for the channel blocker moiety[11–13] and the second was the optimization of the heterocyclic and linker units.[14] The third and current step is to incorporate candidate photoactivatable, fluorescent, biotin, agarose and protein substituents.



R = C≡CCH$_2$OCH$_2$C(O)-linker unit-terminal group

Channel blocker moiety

Four starting materials are used in most of the syntheses considered here. Dithianes **1** and **2** are very potent in blocking the chloride channel, i.e. low IC_{50}s as the concentration for 50% inhibition of ^{3}H ethynylbicycloorthobenzoate (^{3}H EBOB) binding,[15] whereas **3** and **4** are less effective but convenient intermediates for attachment of linker substituents (Fig. 1).

This report describes 23 candidate affinity probes or intermediates in their preparation. It also considers their application in affinity chromatography and immuno-

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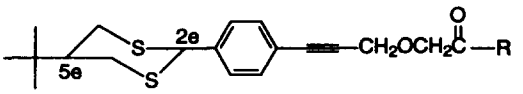
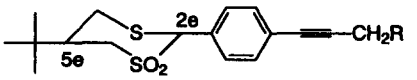


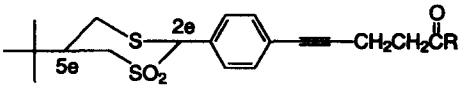
Entry	R	IC_{50}, nM
1	OH	3
2	SCH_2CH_2SH	4
3	$NHCH_2CH_2NH_2$	330
4	$SCH_2CH_2NHCO_2C(CH_3)_3$	14

Figure 1. Structures of dithianes used for synthesis of most of the candidate affinity probes for the NCB site of the GABA-gated chloride channel. Potencies are given for inhibition of ^3H EBOB binding in bovine brain membranes.

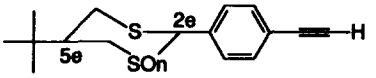
assay. The affinity probes are derived from *tert*-butylethynylphenyldithiane (**22**) as photoactivatable, fluorescent, biotin and agarose/sepharose derivatives (**5–19**) (Scheme 1) or from an analogous dithiane monosulfone (**23**) as hapten–protein/enzyme conjugates (**20–21**) (Fig. 2).



hapten: **20** (R= OTs)
conjugates: **20**-BSA and **20**-HRP

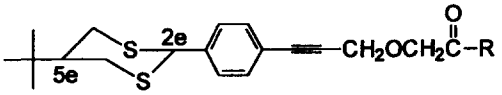


hapten: **21** (R= OH)
conjugates: **21**-BSA, **21**-KLH, and **21**-HRP

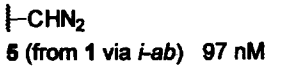


analytes: **22** n= 0, **23** n= 2

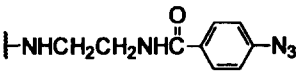
Figure 2. Structures of dithiane monosulfone haptens and hapten–protein/enzyme conjugates and analytes for immunoassay. R substituents for the conjugates are bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) and horseradish peroxidase (HRP).



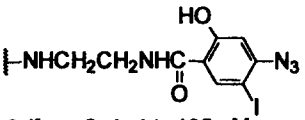
Photoactivable (5-10)



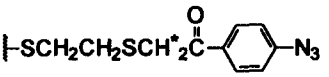
5 (from **1** via *i-ab*) 97 nM



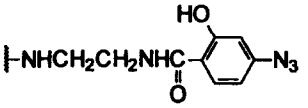
7 (from **3** via *iii*) 850 nM



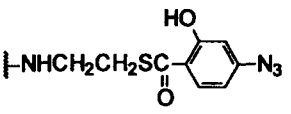
9 (from **8** via *iv*) 105 nM



6, [^{2}H]**6**, [^{3}H]**6** (from **2** via *ii*) 9 nM

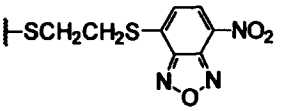


8 (from **3** via *iii*) 2e 170 nM
2a 230 nM

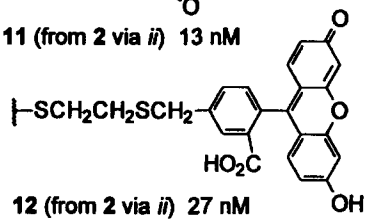


10 (from **4** via *v-ab*) 235 nM

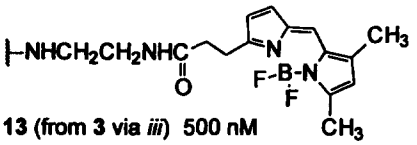
Fluorescent (11-13)



11 (from **2** via *ii*) 13 nM

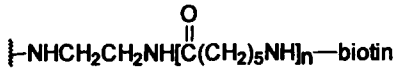


12 (from **2** via *ii*) 27 nM

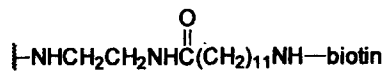


13 (from **3** via *iii*) 500 nM

Biotin (14-16)

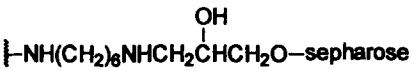


14 (n=1) (from **3** via *iii*) 190 nM
15 (n=2) (from **3** via *iii*) 190 nM



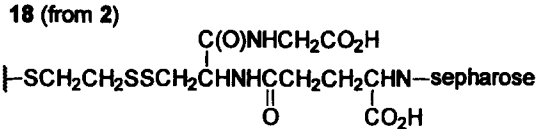
16 (from **3** via *iii*) >10,000 nM

Agarose or sepharose (17-19)



17(from **1**)

18 (from **2**)



19 (from **2**)

Scheme 1. Structures and syntheses of candidate dithiane affinity probes for the NCB site of the GABA-gated chloride channel. Reaction conditions: (i) (a) **1** and excess $(COCl)_2$:CH_2Cl_2:benzene, 0 °C, (b) CH_2N_2:Et_2O, 0 °C; (ii) alkylation of **2** with active halides; (iii) acylation of **3** with NHS carboxylic esters; (iv) iodination by NaI–chloramine-T; (v) (a) **4** and CF_3CO_2H:CH_2Cl_2, (b) NHS-ASA:Et_3N:CH_2Cl_2. Compounds **8**(2*a*) and **8**(2*e*) are axial and equatorial isomers, respectively. Potencies are given as IC_{50} values for inhibition of ^3H EBOB binding in bovine brain membranes.

Chemistry

The photoaffinity ligands incorporate diazoketone (**5**) or azidophenyl (**6–10**) substituents and the fluorescent probes have three types of fluorophores (**11–13**). Biotinylated dithianes **14–16** and agarose or sepharose conjugates **17–19** are candidates for affinity chromatography. Dithiane monosulfone–protein derivatives **20**-BSA, **20**-HRP, **21**-BSA, **21**-HRP and **21**-KLH are hapten–protein conjugates for immunoassay evaluated with analytes **22** and **23**. Relative to synthesis, dithianes **1–4** were converted to candidate affinity probes **5–19**. Diazoketone **5** was synthesized from **1** with excess oxalyl chloride followed by diazomethane.[16] Alkylation of **2** with 4-azidophenacyl bromide (APB), ^{2}H APB, ^{3}H APB, 4-fluoro-7-nitrobenzofurazan and 5-(bromomethyl)fluorescein afforded **6**, ^{2}H **6**, ^{3}H **6** at 7 Ci mmol^{-1}, **11** and **12**, respectively. ^{2}H APB and ^{3}H APB were prepared from APB by alumina-catalyzed deuterium and tritium exchange, respectively, of its enolizable hydrogens.[17] The exchange of deuterium into APB was 80% although that of tritium was only 12%. Acylation of **3** with appropriate *N*-hydroxysuccinimidyl (NHS) carboxylic esters[18] gave **7**, **8**(2*e*) (after chromatographic purification) and **13–16**. Compound **8**(2*a*) was also prepared by acylation of **3**(2*a*) with NHS-4-azidobenzoic acid (NHS-ABA).[19] Iodination of **8** with NaI catalyzed by chloramine-T[20] gave the mono-iodo compound **9**. The dithiane **10** was obtained in the course of removing the *tert*-butoxycarbonyl group of **4** and then coupling with NHS-4-azidosalicylic acid (NHS-ASA).[19] The rearrangement may have occurred after the proposed free amine intermediate was formed;[21] attempts to synthesize the desired R=SCH$_2$-CH$_2$NHC(O)Ar isomer of **10** without rearrangement were not successful. Agarose and sepharose conjugates **17–19** were prepared from **1** and **2** by standard coupling procedures described later. Conjugates **20**-BSA and **20**-HRP were obtained by alkylation of BSA and HRP, respectively, with hapten **20** as the tosylate.[22] Conjugates **21**-BSA, **21**-KLH and **21**-HRP were prepared from hapten **21** by the active ester method.[23,24]

Results

General

The potencies of candidate affinity probes as inhibitors of ^{3}H EBOB binding are given in Scheme 1 and some of them are illustrated in Figure 3. The thiolates derived from **2** were generally more effective than the amidates from **3**.[14]

Photoactivatable dithianes

Diazoketones and aryl azides, which photochemically produce very active carbenes and nitrenes, respectively, are commonly used for photoaffinity labeling.[25] Diazoketone **5** (IC_{50} 97 nM) is improved in potency over its shorter chain analogs (e.g. ArC≡CC(O)CHN$_2$, IC_{50} 690 nM).[12] Azidophenacyl derivative **6**, a potent chloride channel blocker (IC_{50} 9 nM); (Fig. 3), undergoes rapid photolysis at 300 nm in aqueous solution (λ_{max} 302 nm, ε 1.56×10^4); (Fig. 4). To our knowledge ^{3}H **6** is the first high potency tritiated photoactivatable dithiane synthesized for the NCB site of the GABA-gated chloride channel.

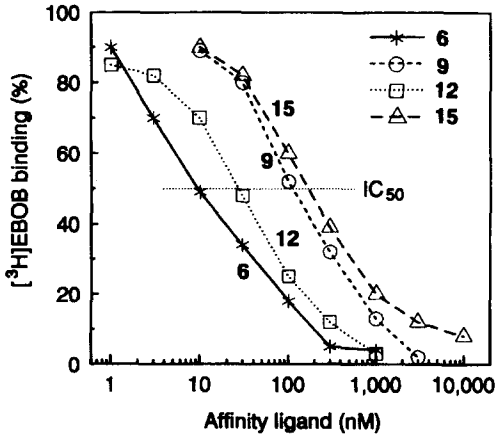


Figure 3. Potency of photoaffinity (**6** and **9**), fluorescent (**12**) and biotinylated (**15**) ligands as inhibitors of ^{3}H EBOB binding in bovine brain membranes.

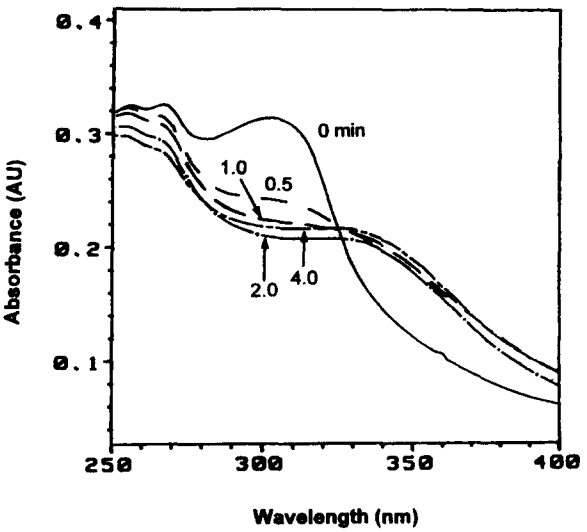


Figure 4. Absorbance spectra showing photolysis of **6** in sodium phosphate buffer (10 mM containing 100 mM NaCl, pH 7.4) at 300 nm. Min refers to photolysis time.

Azidobenzamides **7**, **8**(2*e*) and **9** and a corresponding thioester (**10**) are moderately potent inhibitors with IC_{50}s of 105–850 nM. The potency is increased on introducing an hydroxyl group or both iodo and hydroxyl substituents by 5- and 8.1-fold, respectively. Although the azidosalicylic acid derivative **8** is only moderately potent, the method of preparation used is directly applicable to radiosynthesis from Na^{125}I.[19]

Fluorescent dithianes

Fluorescent probes allow high sensitivity for receptor studies without radiolabeling.[26] Nitrobenzofurazan di-

thiane **11** is a very potent blocker (IC_{50} 13 nM), but its use is limited by low fluorescence yield. The fluorescein derivative **12** combines high potency (IC_{50} 27 nM), strong absorbance (λ_{max} 500 nm and ε 5.8 × 10^4) and high fluorescence yield. Although the BODIPY derivative **13** also has strong absorbance and high fluorescence yield, it is 19-fold less active than **12** in blocking the chloride channel.

Biotinylated dithianes

Biotinylated probes are particularly useful in affinity chromatography.[27] Dithianes **14** and **15** are moderately potent inhibitors (IC_{50} 190 nM) suitable for affinity chromatography, whereas **16** is inactive. Probes **14** and **15** were further examined to see if the dithiane moiety disrupts the ability of the biotin portion to interact with avidin. In an assay for molar effectiveness in displacing the dye 4-hydroxyazobenzene-2-carboxylic acid (HABA) from avidin,[28] the biotinylated dithianes **14** and **15** are almost identical to biotin itself in activity (Fig. 5) so there is no interference between the dithiane and biotin regions. Finally, the binding of avidin to biotinylated probe **14** prevents its dithiane moiety from blocking the receptor (i.e. inhibiting ^{3}H EBOB binding), but this is not the case for biotinylated dithiane **15** with the longer linker substituent (Fig. 6). On this basis, biotinylated dithiane **15** was selected for affinity chromatography with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)-solubilized receptor preparation using the avidin–acrylic bead system eluted with dithiane **1** or GABA (Figure 7). SDS-PAGE analysis revealed several protein bands eluted by dithiane **1** or GABA that fall in the range 51–60 kDa (Fig. 7) as appropriate for $GABA_A$ receptor components purified on a benzodiazapine affinity column.[29] One or

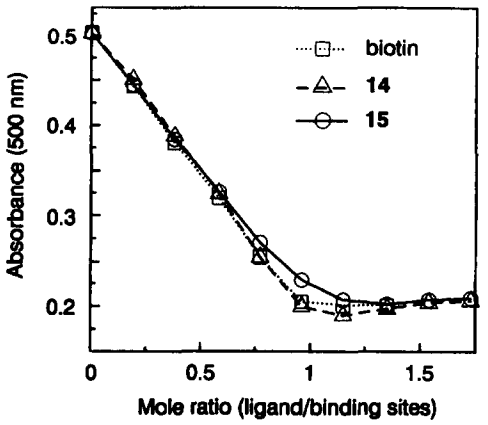


Figure 5. Equivalent effectiveness of biotin and biotinylated dithiane probes **14** and **15** in displacement of dye from avidin.

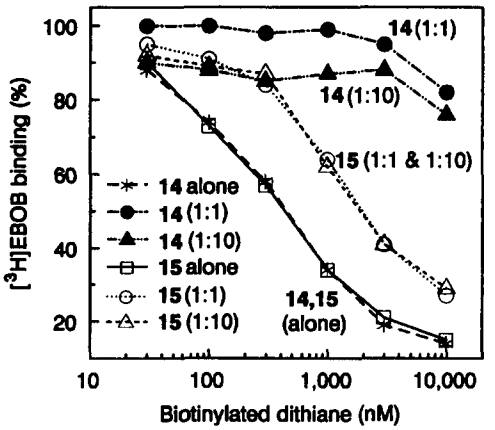


Figure 6. Preincubation of biotinylated dithiane probes with avidin prevents the action of **14** but not of **15** as an inhibitor of ^{3}H EBOB binding in bovine brain membranes. Mole ratios in parentheses (1:1 and 1:10) are biotinylated dithiane: avidin binding site. IC_{50}s of **14** and **15** are *ca* 200 nM after correction for suitable controls.

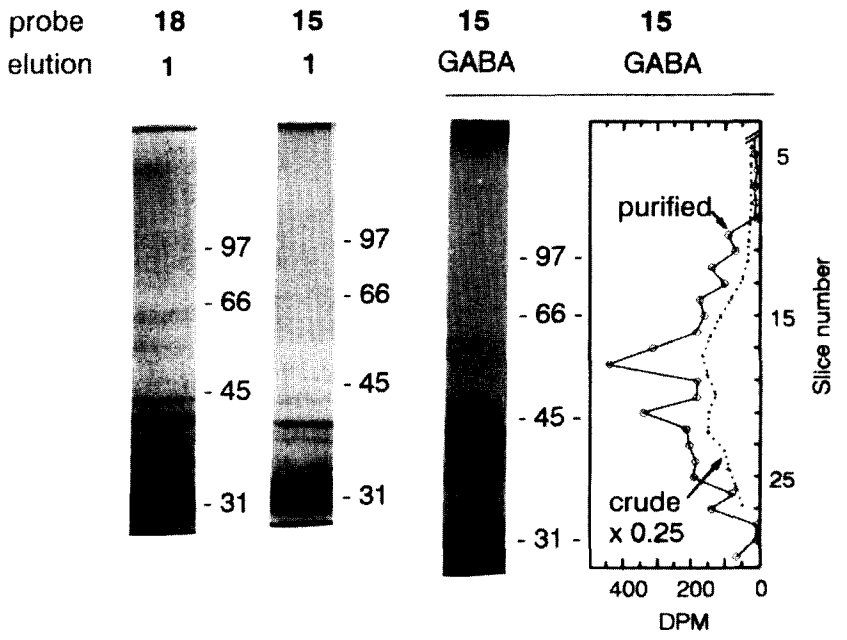


Figure 7. Electrophoretic pattern on Laemmli SDS-PAGE gel of CHAPS-solubilized bovine brain $GABA_A$ receptor purified with either agarose–dithiane **18** as an affinity column or biotinylated dithiane **15** using the avidin–acrylic bead system. Elution was with dithiane **1** or GABA as indicated. The right half of the figure compares proteins detected with silver stain and the $GABA_A$ receptor components detected by ^{3}H flunitrazepam photoaffinity labeling.

more of these proteins appeared to be labeled with ^{3}H flunitrazapam, used as a photoaffinity ligand for the benzodiazapine site prior to SDS-PAGE. The action of GABA in releasing the receptor from the biotinylated dithiane probe may be associated with its effect as a channel opener.[29]

Agarose/sepharose–dithiane conjugates

Agarose- or sepharose-immobilized-dithiane was evaluated for affinity chromatography. Two protein bands (39 and 59 kDa) were removed on passage of the CHAPS-solubilized brain preparation through the affinity column prepared from conjugate **17** (Fig. 8) and the run-through did not bind ^{3}H EBOB or ^{3}H flunitrazepam. The proteins removed by the column are in the range of identified $GABA_A$ receptor proteins.[29] These findings, therefore, are indicative of $GABA_A$ receptor retention on this column. The use of the agarose–dithiane conjugate **18** and elution with dithiane **1** indicated $GABA_A$ receptor components in the eluate (Fig. 7). Sepharose–dithiane conjugate **19** allowed 40% receptor recovery (based on ^{3}H flunitrazepam binding) on elution with cysteine which involves disulfide cleavage, but this resulted in general protein displacement from the column.

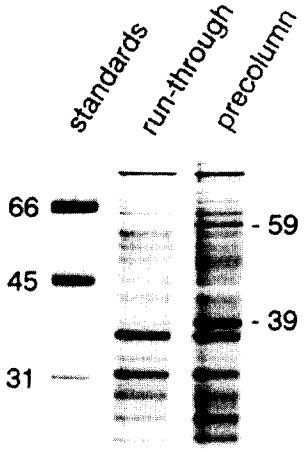


Figure 8. Electrophoretic pattern on Laemmli SDS-PAGE gel of CHAPS-solubilized bovine brain membrane preparation before (precolumn) and after (run through) agarose–dithiane **19** column. Marker proteins are shown. Silver stain.

Protein–dithiane monosulfone conjugates and antisera

Protein conjugates **20**-BSA, **20**-HRP, **21**-BSA and **21**-HRP inhibit ^{3}H EBOB binding with IC_{50}s, as ligand equivalents, of 3.8, 1.1, 0.5 and 0.8 μM, respectively (Fig. 9). Antiserum to the dithiane–monosulfone conjugate **21**-KLH is a strong scavenger for **23**, but not for **22** (Fig. 10).

Discussion

Localization of NCB site in the chloride channel

The orientation of the dithiane affinity probes in the NCB site is most likely to be with the heterocyclic moiety deep within and the terminal substituent more toward the surface of the channel.[12] On this basis the protein–dithiane monosulfone conjugates are inhibitors because the heterocyclic portion can still reach its binding site. The optimal length for the spacer between the ethynyl substituent and the terminal moiety is interpretable from the findings with the biotinylated and agarose/sepharose probes. The biotinylated dithianes, in the presence of avidin, are effective with 22, but not with 15, atoms between the ethynyl moiety and the biotin substituent. The same range of 16–21 atoms is applicable to the agarose/sepharose–dithiane conjugates, whereas in an analogous experiment (data not shown), a linker substituent with 11 atoms was not effective. These observations indicate that 16–22 atoms in the spacer provide useful affinity probes and, therefore, the dithiane or heterocyclic binding site resides at considerable depth within the channel.

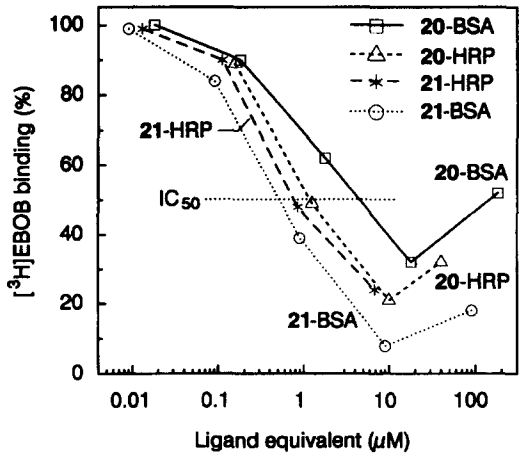


Figure 9. Potency of protein–dithiane monosulfone conjugates as inhibitors of ^{3}H EBOB binding in bovine brain membranes. See Figure 2 for structures of hapten–protein conjugates.

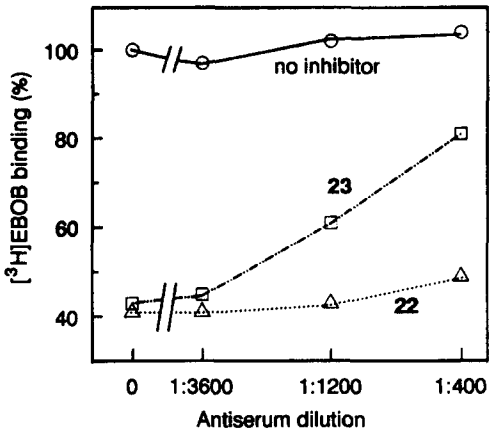


Figure 10. Effect of antiserum to dithiane monosulfone-conjugate **21**-KLH in alleviating inhibition of ^{3}H EBOB binding in bovine brain membranes by dithiane monsulfone **23** (12 nM) but not dithiane **22** (36 nM) at their *ca* IC_{60} concentrations.

Photoactivatable and fluorescent probes

Dithianes ^{3}H **6** and **12** have suitable properties for use in characterizing the chloride channel, bearing in mind that the labeled and fluorophore moieties, respectively,

are at a considerable distance from the channel blocker site.

Affinity chromatography

This study constitutes the first step in applying the NCB site affinity probes to receptor purification. One or more proteins in the region of 51–60 kDa are specifically eluted with GABA or a dithiane and are labeled with ^{3}H flunitrazepam (following photoactivation). These protein(s) are probably components of the $GABA_A$ receptor, but this point remains to be established. *tert*-Butyldithianes are inhibitors of ^{3}H EBOB binding in housefly brain,[30] as well as bovine brain, so the affinity chromatography probes developed here may also be applicable to some insect preparations.

Comparison of dithiane and benzodiazepine probes for affinity chromatography

The findings on biotinylated dithianes and agarose/sepharose–dithiane conjugates differ in several respects from previous studies on benzodiazepine affinity chromatography.[29] Both types of ligands interact at nanomolar levels with the GABA receptor, but the dithianes are normally equilibrated at 37 °C and the benzodiazepines at 4 °C. The dithiane affinity columns are eluted with GABA or a dithiane at 37 °C and the benzodiazepine–agarose column with a benzodiazepine at 4 °C. Thus, the lower temperature and shorter incubation time for the benzodiazepine system make it preferred over the dithiane system for affinity chromatography. However, there are multiple forms of GABA receptors with different subunit composition[31] and benzodiazepine affinity chromatography may favor certain forms over others relative to dithiane affinity chromatography.

Diversity and utility of new affinity probes

These preliminary studies have generated a library of affinity probes useful for characterizing the NCB site of the GABA receptor in mammalian systems. They are potential probes for receptor purification, analysis and heterogeneity studies. They may also be applicable to investigation of the GABA receptor in insect systems.

Experimental

Chemicals

Sources for the chemicals were: APB from Fluka Chemie AG (Buchs, Switzerland); 5-(bromomethyl)-fluorescein (for **12**), *N*-hydroxylsuccinimidyl-4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-propionic acid (BODIPY FL C3-SE) (for **13**), *N*-hydroxylsuccinimidyl-6-((-6-((biotinoyl)amino)hexanoyl)amino) hexanoic acid (biotin-XX, succinimidyl ester) (for **15**) and *N*-hydroxylsuccinimidyl-12-(biotinoylamino)dodecanoic acid (for **16**) from Molecular Probes Inc (Eugene, OR); sulfosuccinimidyl-6-(biotinamido) hexa-noate (NHS-LC-biotin) (for **14**) from Pierce (Rockford, IL); alumina (neutral, activity super I) from ICN Biochemicals, Inc. (Costa Mesa, CA); HRP from Boehringer Mannheim Corp. (Indianapolis, IN); ^{3}H flunitrazepam (85 Ci mmol^{-1}) from Amersham Corp. (Arlington Heights, IL); activated thiol sepharose 4B and EAH sepharose 4B from Pharmacia Biotech Inc. (Alameda, CA); reagents for silver staining from Bio-Rad Laboratories, Inc. (Hercules, CA); avidin–acrylic beads, avidin–agarose 6B, BSA, CHAPS, epoxy-activated agarose 4B, HABA, KLH and poly(ethylene glycol) (PEG) from Sigma Chemical Co. (St. Louis, MO); clonazepam from Richard Squires (Nathan S. Kline Institute for Psychiatric Research, Orangeburg, NY); other chemicals from Aldrich Chemical Co. (St. Louis, MO). Dithianes available from our earlier syntheses were **1–4**, **20–22** and **23**.[12–14] ^{3}H EBOB was available from previous studies.[15] NHS-ABA and NHS-ASA were prepared by the general procedure of Beale *et al.*[19]

Instrumentation, chromatography and characterization of new compounds

The procedures were as previously reported[13,14] except those noted below. Fluorescence spectra were determined with a Perkin–Elmer LS50B luminescence spectrometer. UV–vis spectra were obtained on a HP 8452A diode array spectrophotometer. HPLC involved a Waters 600E instrument with a Model 994 photodiode array detector. Full characterization data are given on representative compounds **5**, **6** and **9**, but only partial ^{1}H and ^{13}C NMR data and spectral assignments for the R substituents are described for **7**, **8**(2*a*), **8**(2*e*) and **10–16** since the other regions of these molecules gave spectra consistent with those of the representative compounds.

Photolysis

Aqueous solutions of **5**, **6**, **7**, **9** and **10** in quartz cuvettes at 21 °C were placed 3 cm from four UV lamps in a Rayonet photochemical reactor (The Southern New England Ultraviolet Co., Hamden, CT). The photoreactivity of **6** (Fig. 4) is typical of that for the other compounds.

Preparation of membranes and solubilized receptor

Bovine brain membranes (prepared from 25 g of tissue as in our earlier description[13]) were suspended in 60 mL of assay buffer (100 mM NaCl–10 mM Na phosphate pH 8.0) containing 20 mM CHAPS, 2 mM benzamidine hydrochloride, 0.1 mM benzethonium chloride, 1 mM EDTA, 20 mg mL^{-1} bacitracin, 0.1 mM phenylmethylsulfonyl fluoride and 0.02% (wt/vol) NaN_3. This suspension was gently stirred for 30–35 min, then centrifuged at 130,000 *g* for 70 min, each at 4 °C. The solubilized receptor in the supernatant without dialysis was used the same day or stored at –70 °C for several months, but with a slight decrease of binding activity.

Receptor assays – ^{3}H EBOB and ^{3}H flunitrazepam

^{3}H EBOB binding assays with membrane preparations were as previously reported.[13,14] The PEG precipitation and filtration procedure of Seifert and Casida[32] was modified for ^{3}H EBOB assay with the CHAPS-solubilized preparation (0.2 mg protein per assay) and purified receptor (protein concentration not determined). After incubation, γ-globulin (30 μL of 3.3% solution in assay buffer) and PEG (0.3 mL of 36% aqueous solution) were added to the assay mixture and vigorously vortexed. After 10 min at 0 °C the mixture was filtered through Whatman GF/C glass fiber filters, which were then rinsed with ice-cold assay buffer containing 6.7% PEG (3 × 4 mL).

The ^{3}H flunitrazepam binding assay procedure differed from that described for ^{3}H EBOB in the following ways: ^{3}H flunitrazepam (1.2 nM) replaced ^{3}H EBOB (0.57 nM); clonazepam (1.5 mM) was used instead of 4-*sec*-butyl-1-(4-cyanophenyl)-2,6,7-trioxabicyclo(2.2.2)-octane (2 μM) to correct for non specific binding; incubation was for 30 min at 4 °C instead of 90 min at 37 °C.

Affinity chromatography and photoaffinity labeling

Purification of $GABA_A$ receptor complex via biotinylated dithiane 15. The solubilized receptor (from *ca* 20 g of bovine brain) in 160 mL of KBr–assay buffer (0.5 M KBr in assay buffer) was incubated with biotinylated dithiane **15** (0.4 μmol in 205 μL DMSO) by gentle shaking for 60–75 min at 37 °C. Avidin–acrylic beads (100 units) pre-equilibrated with assay buffer were then added and incubated for 30 min at 37 °C. The mixture was introduced into a Bio-Rad Econo-column (1 cm ID) and cycled twice at a flow rate of 5 mL min^{-1}. After the column was washed with 80 mL of 0.5 M KBr-assay buffer, the bound receptor complex was eluted by compound **1** (0.1 mM) or GABA (10 mM) dissolved in 0.5 M KBr-assay buffer at room temperature. The combined eluents were desalted and concentrated with the Centricon-10 concentrator (Amicon, Inc., Beverly, MA) to 200–300 μL in assay buffer.

Purification of $GABA_A$ receptor complex via sepharose–dithiane conjugates 17 and 19 and agarose–dithiane conjugate 19. Conjugate **18** (10 mL of swollen gel) was incubated with solubilized receptor preparation (from 2 g of bovine brain) in 15 mL of assay buffer for 90 min at 37 °C, then washed with 60 mL of assay buffer after loading onto a column; the run-through was assayed with ^{3}H EBOB and ^{3}H flunitrazepam and analyzed by SDS-PAGE. The procedure for agarose conjugate **18** differed from that described for biotinylated dithiane **15** in that 15 mL of the swollen gel **18** was used with one cycle for loading the column. The procedure for trapping of receptor with conjugate **19** was the same as for **17**, but the bound proteins were eluted by incubation of the gel with 5 mL of 20 mM L-cysteine in assay buffer overnight at 4 °C and the cysteine eluates were assayed for ^{3}H flunitrazepam binding.

Photoaffinity labeling with ^{3}H flunitrazepam. The method of Bureau and Olsen[33] was modified as follows: the solubilized receptor preparation (0.5 mg protein) or purified receptor proteins eluted with GABA from the **15**-avidin–acrylic bead column were incubated with ^{3}H flunitrazepam (47 nM) without or with clonazepam (50 mM) in 0.5 mL of assay buffer for 1 h at 4 °C, followed by irradiation for 15 min under 350 nm UV lights as described above.

Immunization of rabbits

The immunization protocol was that of Li *et al.*[34] with slight modifications. Three female New Zealand white rabbits (2–3 kg) each initially received 100 μg (protein equivalent) of **21**-KLH [mixed in 1 mL of phosphate-buffered saline (PBS) and Freund's complete adjuvant (1: 1)] administered subcutaneously in multiple spots on the back, followed by 4 boosts with 200 μg of appropriate immunogen [in 1 mL of PBS and Freund's incomplete adjuvant (1: 1)] every 3 weeks. At the 10th day after each boost, about 10 mL of blood was collected to monitor the antibody production. When the titer was sufficiently high, blood was collected by heart puncture. Serum was isolated by centrifugation and NaN_3 added to a final concentration of 0.02% with storage at –70 °C.

Other methods

Protein was determined by the method of Bradford.[35] SDS-PAGE was performed by the procedure of Laemmli.[36] Silver staining involved the technique of Willoughby and Lambert.[37]

Synthesis

Compound 5. To a solution of **1** (0.16 mmol) in dry CH_2Cl_2 (4 mL) and benzene (7 mL) precooled on ice was added excess oxalyl chloride followed by stirring for 1 h at room temperature. After removal of solvent, the residue was cooled on ice and treated with excess diazomethane in ether and stirred for 20 min at room temperature. The product was purified by flash chromatography (hexane:EtOAc 7:3). Yield, 56%; ^{1}H NMR ($CDCl_3$) δ 0.96 (9H, *s*, $3CH_3$), 1.76 (1H, *tt*, *J* = 2.5, 11.1 Hz, H-5*a*), 2.83 (2H, *dd*, *J* = 11.1, 14.1 Hz, H-4*a*/6*a*), 2.97 (2H, *dd*, *J* = 2.5, 14.1 Hz, H-4*e*/6*e*), 4.16 (2H, *s*, CH_2O), 4.44 (2H, *s*, OCH_2), 5.12 (1H, *s*, H-2*a*), 5.78 (1H, *s*, CHN_2), 7.39 (2H, *d*, *J* = 8.3 Hz, aromatic), 7.43 (2H, *d*, *J* = 8.3 Hz, aromatic); ^{13}C NMR ($CDCl_3$) δ 27.3 (×3), 33.5 (×2), 33.9, 46.2, 50.9, 53.4, 59.5, 73.2, 84.1, 87.1, 122.3, 127.8 (×2), 132.1 (×2), 139.0; FTIR (KBr) 2111 (s, CN_2), 1633 (s, C=O), 1351 (s, CN_2) cm^{-1}; LRMS (EI) 388 (M^+, 2).

Compounds 6, ^{2}H 6, ^{3}H 6, 11 and 12. Equimolar **2** and APB (for **6**), ^{2}H APB (for ^{2}H **6**), 4-fluoro-7-nitrobenzofurazan (for **11**) or 5-(bromomethyl)fluorescein (for **12**) were stirred with 1.1–6 eq. of triethylamine (Et_3N) in dry CH_2Cl_2 (2 h for **6** and 10 min for **11**), in benzene (20 min for ^{2}H **6**), or in DMF (overnight for **12**). After

evaporation of solvent, the products were chromatographically purified.

6: yield 100 %; ^{1}H NMR (C_6D_6) δ 0.57 (9H, *s*, $3CH_3$), 1.66 (1H, *tt*, *J* = 2.0, 10.5 Hz, H-5*a*), 2.38 (2H, *dd*, *J* = 10.5, 13.5 Hz, H-4*a*/6*a*), 2.56 (4H, *m*, CH_2S and H-4*e*/6*e*), 2.96 (2H, *t*, *J* = 7.4 Hz, SCH_2), 3.33 (2H, *s*, SCH_2), 3.98 (2H, *s*, CH_2O), 4.07 (2H, *s*, OCH_2), 4.89 (1H, *s*, H-2*a*), 6.51 (2H, *d*, *J* = 8.7 Hz, aromatic), 7.29 (2H, *d*, *J* = 8.2 Hz, aromatic), 7.47 (2H, *d*, *J* = 8.2 Hz, aromatic), 7.61 (2H, *d*, *J* = 8.7 Hz, aromatic); ^{13}C NMR (C_6D_6) δ 27.2 (×3), 27.6, 32.3, 33.6 (×2), 36.7, 39.5, 46.4, 51.3, 59.7, 74.2, 84.9, 87.5, 119.0 (×2), 122.9, 130.9 (×2), 132.2, 132.5 (×2), 140.0, 144.9, 192.2, 198.3; HRMS (FAB) calcd $C_{29}H_{33}N_3O_3S_4H^+$ 600.1483, found 600.1493.

^{2}H **6**: ^{2}H APB was prepared by alumina-catalyzed deuterium exchange. Alumina N-super I (0.5 g) and deuterium oxide (20 μL) were mixed by swirling. After 2 h, APB (21 μmol) was added in dry benzene (0.5 mL), swirled and allowed to sit for 40 min. ^{2}H APB in dry benzene extract (5 × 0.5 mL) was directly used for the synthesis of ^{2}H **6**: partial ^{1}H NMR (C_6D_6) δ 2.96 (2H, *t*, *J* = 7.4 Hz, SCH_2), 3.34 [0.4H corresponding to 80% ^{2}H exchange, *s*, $SCH^*_2C(O)$], 3.98 (2H, *s*, OCH_2), 4.07 (2H, *s*, CH_2O).

^{3}H **6**: After a flask containing alumina (neutral, super I, 0.5 g) was evacuated, it was immersed in a liquid nitrogen bath and tritium oxide (20 μL, about 60 Ci) generated from tritium gas over platinum(IV) oxide was introduced by vacuum transfer. Upon removal of the bath, the flask was filled with dry nitrogen and the alumina was equilibrated for 1.5 h at room temperature. APB (21 μmol) in dry benzene (5 mL) was added by needle transfer and allowed to sit for 35 min. ^{3}H APB was extracted with dry benzene (3 and 2 mL) and the extract placed in a flask containing anhydrous Na_2SO_4 (0.6 g) by needle transfer and then transferred to another flask with **2** (27 μmol) and Et_3N (30 μmol) in dry benzene (0.4 mL) and stirred for 3 h at room temperature. The mixture was lyophilized to give 395 mCi of residue. After flash chromatographic purification (hexane:EtOAc 4:1), ^{3}H **6** was further purified by HPLC with a Vydac C_4 column (Separations Group, Hesperia, CA; 5 μm, 0.46 × 5 cm) with water:acetonitrile (4:1) at a flow rate of 1.5 mL min^{-1}. ^{3}H **6**: specific activity 7 Ci mmol^{-1} based on radio HPLC analysis; ^{3}H NMR (C_6D_6) δ 3.34 [*s*, $SC^3H_3C(O)$].

11: yield, 75%; partial ^{1}H NMR ($CDCl_3$) δ 3.26 (2H, *m*, SCH_2), 3.42 (2H, *m*, CH_2S), 7.63 (1H, *d*, *J* = 8.0 Hz, aromatic), 8.59 (1H, *d*, *J* = 8.0 Hz, aromatic); partial ^{13}C NMR ($CDCl_3$) δ 26.6, 31.1, 121.3, 128.7, 130.7, 131.8, 139.5, 142.5; HRMS (FAB) calcd $C_{27}H_{29}N_3O_5S_4^+$ 603.0990, found 603.0991. In ethanol solution **11** has UV–vis absorption peaks at 200, 250 and 412 nm and gives low fluorescence.

12: yield 36%; partial ^{1}H NMR ($CDCl_3$:CD_3OD) δ 2.71 (2H, *m*, SCH_2), 3.17 (2H, *s*, SCH_2), 3.94 (2H, *t*, *J* = 7.4 Hz, CH_2S), 6.62 (2H, *br*, aromatic), 6.70 (2H, *br*, aromatic), 6.84 (2H, *br*, aromatic), 7.15 (1H, *br*, aromatic), 7.70 (1H, *br*, aromatic), 8.03 (1H, *br*, aromatic); HRMS (FAB) calcd $C_{42}H_{40}O_7S_4H^+$ 785.1735, found 785.1748; absorption λ_{max} 500 nm (ε 5.8 × 10^4), emission λ_{max} 520 nm with intensity of 250 at a concentration of 20 nM in ethanol.

Compounds ***7***, ***8(2a)***, ***8(2e)***, ***13–16***. Equimolar **3** or **3**-axial isomer [for **8**(2*a*)] and NHS-carboxylic acid derivatives and 1.1–2.0 eq. of Et_3N were stirred for 1–3 h (or overnight for **13** and **16**) at room temperature in dry CH_2Cl_2 [for **7**, **8**(2*a*) and **8**(2*e*)] or in dry DMF (for **13–16**). After removal of solvents, products were purified by flash chromatography.

7: yield 100%; partial ^{1}H NMR ($CDCl_3$) δ 3.58 (4H, *m*, $2CH_2N$), 7.03 (2H, *d*, *J* = 8.6 Hz, aromatic), 7.17 (1H, *br*, NH), 7.54 (1H, *br*, NH), 7.82 (1H, *d*, *J* = 8.6 Hz, aromatic); partial ^{13}C NMR ($CDCl_3$) δ 39.0, 41.7, 118.9 (×2), 128.8 (×2), 130.5, 143.2, 171.3; FTIR (KBr) 3405 (s, NH), 2123 (s, N_3), 1638 (s, C=O) cm^{-1}.

8(2*a*): yield 100%; ^{1}H NMR ($CDCl_3$) δ 0.87 (9H, *s*, $3CH_3$), 1.84 (1H, *tt*, *J* = 3.0, 10.5 Hz, H-5*a*), 2.61 (2H, *dd*, *J* = 10.5, 13.5 Hz, H-4*a*/6*a*), 2.72 (2H, *dd*, *J* = 3.0, 13.5 Hz, H-4*e*/6*e*), 3.59 (4H, *br*, $2CH_2N$), 4.17 (2H, *s*, CH_2O), 4.48 (2H, *s*, OCH_2), 4.86 (1H, *s*, H-2*e*), 6.51 (1H, *dd*, *J* = 3.0, 9.0 Hz, aromatic), 6.61 (1H, *d*, *J* = 3.0 Hz, aromatic), 7.14 (1H, *br*, NH), 7.42 (2H, *d*, *J* = 9.0 Hz, aromatic), 7.44 (1H, *d*, *J* = 9.0 Hz, aromatic), 7.74 (2H, *d*, *J* = 9.0 Hz, aromatic), 7.95 (1H, *br*, NH); LRMS (FAB) 568 (MH^+).

8(2*e*): yield 100%; ^{1}H NMR ($CDCl_3$) δ 0.95 (9H, *s*, $3CH_3$), 1.75 (1H, *tt*, *J* = 2.4, 11.2 Hz, H-5*a*), 2.83 (2H, *dd*, *J* = 11.2, 12.4 Hz, H-4*a*/6*a*), 2.97 (2H, *dd*, *J* = 2.4, 12.4 Hz, H-4*e*/6*e*), 3.55 (4H, *br*, $2CH_2N$), 4.13 (2H, *s*, CH_2O), 4.44 (2H, *s*, OCH_2), 5.11 (1H, *s*, H-2*a*), 6.47 (1H, *dd*, *J* = 3.0, 9.0 Hz, aromatic), 6.58 (1H, *d*, *J* = 3.0 Hz, aromatic), 7.17 (1H, *br*, NH), 7.35 (2H, *d*, *J* = 9.0 Hz, aromatic), 7.42 (2H, *d*, *J* = 9.0 Hz, aromatic), 7.45 (1H, *d*, *J* = 9.0 Hz, aromatic), 7.95 (1H, *br*, NH); partial ^{13}C NMR ($CDCl_3$) δ 38.8, 41.6, 107.8, 109.9, 111.0, 127.7, 145.5, 162.8, 171.8; FTIR (KBr) 3417 (sh, OH), 3347 (sh, NH), 2111 (s, N_3), 1644 (s, C=O) cm^{-1}; LRMS (FAB) 568 (MH^+).

13: yield 87%; partial ^{1}H NMR ($CDCl_3$) δ 2.25 (3H, *s*, CH_3), 2.56 (3H, *s*, CH_3), 2.64 (2H, *t*, *J* = 7.4 Hz, CH_2), 3.27 (2H, *t*, *J* = 7.4 Hz, CH_2), 3.35 (4H, *m*, $2CH_2N$), 6.11 (1H, *s*, aromatic), 6.17 (1H, *br*, NH), 6.27 (1H, *d*, *J* = 4.0 Hz, aromatic), 6.87 (1H, *d*, *J* = 4.0 Hz, aromatic), 6.94 (1H, *br*, NH), 7.08 (1H, *s*, aromatic); partial ^{13}C NMR ($CDCl_3$) δ 11.3, 15.0, 24.9, 35.8, 39.3, 39.6, 117.4, 120.5, 123.8, 128.1, 170.0; HRMS (FAB) calcd $C_{35}H_{42}BFN_4O_3S_2H^+$ 661.2854, found 661.2837; absorption λ_{max} 506 nm and emission λ_{max} 513 nm in ethanol.

14: yield 90%; partial ^{1}H NMR ($CDCl_3$:CD_3OD) δ 1.31–1.67 (12H, *m*, $6CH_2$), 2.19 (4H, *m*, $2CH_2$), 2.75 (2H, *m*,

biotin CH_2S), 3.19 (3H, *m*, CH_2N and CHS), 3.38 (4H, *m*, $2CH_2N$), 4.31 (1H, *m*, CHN), 4.52 (1H, *m*, CHN); partial ^{13}C NMR ($CDCl_3$:CD_3OD) δ 24.9, 25.3, 26.1, 27.9, 28.0, 28.7, 35.5, 35.9, 38.8, 38.9, 39.1, 40.3, 55.4, 60.0, 61.8, 170.6, 173.7, 174.6; HRMS (FAB) calcd $C_{37}H_{55}N_5O_5S_3H^+$ 746.3444, found 746.3447.

15: Yield 39%; partial ^1H NMR ($CDCl_3$:CD_3OD) δ 1.26–1.71 (18H, *m*, $9CH_2$), 2.17 (6H, *m*, $3CH_2$), 2.75 (2H, *m*, biotin CH_2S), 3.18 (5H, *m*, $2CH_2N$ and CHS), 3.37 (4H, *m*, $2CH_2N$), 4.31 (1H, *m*, CHN), 4.52 (1H, *m*, CHN); partial ^{13}C NMR ($CDCl_3$:CD_3OD) δ 24.9, 25.0, 25.3, 26.0 (×2), 27.9, 28.1, 28.6 (×2), 35.5, 35.9 (×2), 38.7, 38.9 (×2), 39.0, 40.3, 55.4, 60.1, 61.8, 170.6, 173.8, 174.1, 174.7; HRMS (FAB) calcd $C_{43}H_{66}N_6O_6S_3H^+$ 859.4284, found 859.4230.

16: Yield 8%; partial ^1H NMR ($CDCl_3$:CD_3OD) δ 1.26–1.63 (24H, *m*, $12CH_2$), 2.18 (2H, *t*, *J* = 7.2 Hz, CH_2), 2.20 (2H, *t*, *J* = 6.1 Hz, CH_2), 2.77 (2H, *m*, biotin CH_2S), 3.19 (3H, *m*, CH_2 and CHS), 3.37 (4H, *m*, $2CH_2N$), 4.31 (1H, *m*, CHN), 4.52 (1H, *m*, CHN); partial ^{13}C NMR ($CDCl_3$:CD_3OD) δ 25.3, 25.4, 26.6, 27.8, 28.1, 28.9 (×4), 29.1 (×2), 35.4, 36.0, 38.6, 38.7, 39.2, 39.9 (×2), 55.3, 60.0, 61.8, 170.7, 173.9, 174.7; HRMS (FAB) calcd $C_{43}H_{67}N_5O_5S_3H^+$ 830.4383, found 830.4373.

Compound 9. To a solution of **8** (0.02 mmol) and NaI (0.06 mmol) in DMF (0.5 mL) was added chloramine-T (0.04 mmol) with stirring for 1 h at room temperature. The product was diluted with water, acidified with aqueous HCl (1 N), extracted with EtOAc and purified by flash chromatography (hexane:EtOAc:acetic acid 70:30:3). Yield 82%; ^1H NMR ($CDCl_3$) δ 0.96 (9H, *s*, $3CH_3$), 1.75 (1H, *tt*, *J* = 2.4, 11.2 Hz, H-5*a*), 2.83 (2H, *dd*, *J* = 11.2, 12.4 Hz, H-4*a*/6*a*), 2.96 (2H, *dd*, *J* = 2.4, 12.4 Hz, H-4*e*/6*e*), 3.57 (4H, *br*, $2CH_2N$), 4.16 (2H, *s*, CH_2O), 4.45 (2H, *s*, OCH_2), 5.11 (1H, *s*, H-2*a*), 6.72 (1H, *s*, aromatic), 7.14 (1H, *br*, NH), 7.36 (2H, *d*, *J* = 7.5 Hz, aromatic), 7.42 (2H, *d*, *J* = 7.5 Hz, aromatic), 7.87 (1H, *s*, aromatic), 7.96 (1H, *br*, NH); ^{13}C NMR ($CDCl_3$) δ 27.3 (×3), 33.5 (×2), 33.9, 38.8, 41.6, 46.2, 50.9, 59.5, 68.9, 83.7, 87.4, 107.6, 122.0, 126.4, 127.9 (×2), 129.7, 132.1 (×2), 137.4, 139.2, 146.9, 162.9, 168.7, 171.8; HRMS (FAB) calcd $C_{28}H_{32}IN_5O_4S_2H^+$ 694.1019, found 694.1002.

Compound 10. Compound **4** (0.42 mmol) in CH_2Cl_2: trifluoroacetic acid (4:1, 5 mL) was stirred for 10 min, then aqueous NaOH (1 N, 20 mL) was added followed by extraction with CH_2Cl_2 (3 × 5 mL) and drying (Na_2SO_4). To the extract NHS-ASA (0.5 mmol) and excess Et_3N were added with stirring for 2.5 h followed by washing with aqueous HCl (0.1 N), then saturated aqueous NaCl. After drying over Na_2SO_4, the products were purified by flash chromatography (hexane: EtOAc:acetic acid 70:30:3). Yield 10%; partial ^1H NMR ($CDCl_3$) δ 3.26 (2H, *t*, *J* = 6.0 Hz, CH_2S), 3.60 (2H, *m*, CH_2N), 6.53 (1H, *dd*, *J* = 3.0, 9.0 Hz, aromatic), 6.60 (1H, *d*, *J* = 3.0 Hz, aromatic), 7.80 (1H, *d*, *J* = 9.0 Hz, aromatic), 11.2 (1H, *br*, OH); partial ^{13}C NMR ($CDCl_3$) δ 28.3, 38.7, 107.6, 110.8, 116.9, 130.8, 147.9, 161.1, 169.7; LRMS (FAB) 585 (MH^+).

Agarose/sepharose–dithiane conjugates 17–19. Compound **17**: **1** (0.14 mmol) was activated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride and then immobilized on EAH sepharose 4B (20 ml of suspension) by the general procedure provided by Pharmacia Biotech Inc.

18: Compound **2** (0.41 mmol) was coupled to epoxy-activated thiol agarose 4B (1 g of freeze-dried powder) by the procedure of Simons and Vander Jagt.[38]

19 was prepared by coupling **2** (2 mg, 4.4 μmol) with activated thiol sepharose 4B (1 g of freeze-dried powder) according to the procedure of Stuchbury *et al.*[39]

Protein–dithiane monosulfone conjugates and antisera

Protein conjugate 20-BSA. Compound **20** (35 μmol) in dioxane (4 mL) was added to BSA (25 mg) in 0.5 M carbonate buffer pH 10.7 (4 mL) and stirred at room temperature for 6 h.

Protein conjugates 21-BSA and 21-KLH. Compound **21** (25 μmol), *N*-hydroxysuccinimide (27 μmol) and 1,3-dicyclohexylcarbodiimide (27 μmol) in anhydrous DMF (0.5 mL) were stirred at ambient temperature for 3 h. After filtration of the precipitate, the filtrate was added to BSA (25 mg) or KLH (25 mg) in water:DMF (2:1) (6 mL). The reaction mixture was stirred for 30 min at room temperature, then overnight at 4 °C. After the above protein conjugates were extensively dialyzed against PBS (10 × 4 L, 7 days at 4 °C), the ligand–protein conjugates were determined by UV–vis spectra and then stored in PBS at –70 °C for later use. Antisera to dithiane–monosulfone conjugate **21**-KLH was assayed for alleviating inhibition of ^3H EBOB binding in bovine brain membrane by **22** and **23** at their IC_{60} concentrations. All incubation components were mixed quickly at 25 °C and immediately incubated for 90 min at 37 °C before filtration to determine ^3H EBOB binding.

Enzyme conjugates 20-HRP and 21-HRP. To prepare **20**-HRP, **20** (1.5 mg) in DMF (130 μL) was added to HRP (2 mg) in 0.13 M $NaHCO_3$ (3 mL), followed by stirring for 1 h at room temperature then overnight at 4 °C. Compound **21**-HRP was prepared by the procedure of Schneider and Hammock[24]. Compounds **20**-HRP and **21**-HRP were dialyzed against 0.13 M $NaHCO_3$ (10 × 2 L) for 3 days at 4 °C and stored in the solution of $NaHCO_3$ buffer and ethylene glycol (1:1) at –20 °C.

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