Synthesis of Conjugates of 6-Aminophenanthridine and Guanabenz, Two Structurally Unrelated Prion Inhibitors, for the Determination of Their Cellular Targets by Affinity Chromatography

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The synthesis of affinity matrices for 6-aminophenanthridine (6AP) and 2,6-dichlorobenzylidenaminoguanidine (Guanabenz, GA), two unrelated prion inhibitors, is described. In both cases, the same simple spacer, ε-aminocaproylaminopentanol, was introduced by a Mitsunobu reaction and the choice of the anchoring position of the linker was determined by the study of the residual antiprion activity of the corresponding 6AP or GA conjugates. Very recently, these two affinity matrices were used for chromatography assays leading to the identification of ribosome (via the rRNA) as a common target of these two antiprion drugs. Here, we show, using competition experiments with Quinacrine (QC) and Chlorpromazine (CPZ), two other antiprion drugs, that QC, but not CPZ, may also directly target the rRNA.

INTRODUCTION

Prion-based diseases are transmissible and yet incurable fatal neurodegenerative disorders (*1*). Among these diseases are Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathies in cattle, and Scrapie in sheep and goats. These diseases are associated with neuronal cell death, which leads to characteristic "spongiform" vacuolation of the brain, and are therefore termed spongiform encephalopathies. According to the "protein-only" hypothesis, prions are mostly if not solely composed of an abnormally folded form (PrP^{Sc}) of the PrP protein (PrP^{C}), a glycosyl-phosphatidyl inositol (GPI) anchored protein normally expressed at the surface of a number of cell types including in particular neurons. Transmissibility necessitates the conversion of host PrP^{C} by exogenous PrP^{Sc}. PrP^{Sc} isoform displays a pronounced protease resistance, shows an increase in β-sheet structures, and forms amyloid fibers. Despite intensive works devoted to the study of prions and their mechanisms of propagation, many fundamental questions related to the mechanism of the infection and subsequently to the mechanisms of action of known antiprion compounds remain unanswered. On the basis of the assumption that PrP^{Sc} corresponds to (or at least is part of) the pathogenic entity, various approaches aimed at reducing PrP^{Sc} or PrP^{C} levels for the development of prion disease therapies are currently being explored (comprehensively reviewed in ref *2*). A proof of concept for these approaches has been made recently by showing that depleting PrP^{C} from neurons of prion-infected mice in which the *Prnp* gene can be turned off, not only prevented progression of disease, but also reversed spongiosis and early cognitive and neurophysiological dysfunctions (*3, 4*). Therefore, approaches leading to reduction of endogenous PrP^{C} and/or PrP^{Sc} levels may well be effective after the appearance of the neurological symptoms. Among these approaches are identifications of pharmacological compounds promoting PrP^{Sc} clearance. Some of these identifications are based on the use of cell-free systems (*5–8*), whereas others are based on the use of mammalian cells chronically infected with prions (*9–12*) and reviewed in ref *13*. All these assays are time- and money-consuming, in particular, because experiments have to be carried out in highly secured laboratories. For these reasons, we recently developed a rapid and economical budding yeast (*Saccharomyces cerevisiae*) based two-step assay to screen for antiprion molecules (*14, 15*). This assay allows easy detection of compounds active against two yeast prions and has led, among others, to the isolation of 6-aminophenanthridine (6AP) **1a** and 2,6-dichlorobenzylidenaminoguanidine (Guanabenz, GA) **1b** (Figure 1). This yeast-based assay was validated for the isolation of drugs active against mammalian prions, since most of the active compounds isolated, including 6AP and GA, turned out to also be active against mammalian prions in mammalian cell-based assays mentioned above (*16*). In addition, both 6AP and GA exhibited significant activity in a mouse model for scrapie (*17*).

Although direct interaction with the prion protein PrP^{C} has been established by NMR spectroscopy (*18*) or by surface plasmon resonance (*19*) for some drugs, this has not been evaluated with most of the known antiprion agents. Recently, using immobilized 9-aminoacridine, Prusiner's group was able to discriminate PrP^{C} and PrP^{Sc} in an affinity chromatography assay (*20*). Moreover, even if some of the compounds are known to bind PrP, it is not established whether their antiprion activities exclusively rely on this binding ability. Therefore, the mechanism of action of most antiprions remains unclear, and at least two different mechanisms can be envisioned: drugs can either act in *cis* directly on prion aggregates or in *trans* on cellular elements required for prion propagation. In addition, identification of these unknown biological mechanisms may provide new

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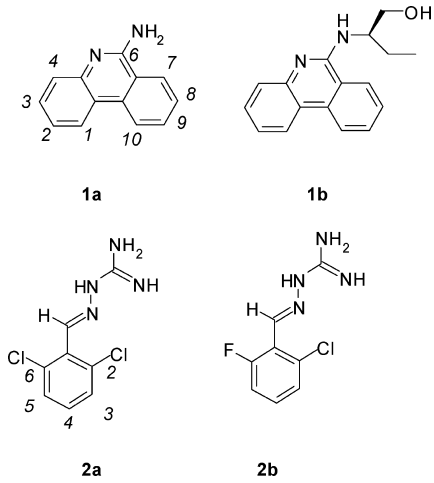


Figure 1. 1a, 6-aminophenanthridine (6AP) and **2a**, 2,6-dichlorobenzylidenaminoguanidine: Guanabenz (GA), two antiprion drugs active against both yeast and mammalian prions and their respective inactive controls, **1b** (6APi) and **2b** (GAi).

insights for other neurodegenerative disorders characterized by the occurrence of protein misfolding as observed in prion diseases (*21*).

For these reasons, identification of these mechanisms using reverse screening approaches is of particular interest. Several comprehensive methods exist to decipher cellular mechanisms targeted by small chemical compounds, including affinity chromatography on immobilized drugs.

Affinity chromatography has been used in the determination of the molecular targets of several compounds. Most of them are kinase inhibitors such as flavopiridol (*22*), purvalanol (*23*), (*R*)-roscovitine (*24*), gefitinib (*25*), and SU6668 (*26*). Affinity chromatography has also been performed with the immunosuppressor FK506 (*27*) and geldanamycin, which was therefore shown to be a specific inhibitor of HSP 90 (*28*).

As a typical example, the determination of the molecular targets of (*R*)-roscovitine led, apart from the expected cyclin-dependent kinases, to the identification of pyridoxal kinase, an unexpected target. This was confirmed by X-ray crystallography, and surprisingly, roscovitine appeared to bind the pyridoxal pocket rather than the ATP binding site (*29*).

In affinity chromatography experiments, a spacer arm is introduced between the compound of interest and the solid supports with the aim of avoiding steric hindrance. The structure of the spacer arm matters considerably. Polyoxyethylenic linkages are generally used. A carboxylic acid group is often introduced on the active molecules. Therefore, the introduction of the spacer on the bioactive compound is frequently achieved by a multiple-step procedure. The position of the linkage is critical, as the conjugate should, at least partially, retain biological activity. Linkage should be introduced at several positions of the molecule under study and the biological activity of the resulting conjugates tested. If available, structure−activity relationships provide useful information on the positions of linkage which are more likely to allow molecular recognition by the biological targets (for a review, see ref *30*).

In this paper, we report the synthesis of affinity matrices for the two unrelated antiprion drugs cited above: 6-aminophenanthridine (6AP) and 2,6-dichlorobenzylidenaminoguanidine (guanabenz, GA) by immobilization of the conjugates on preactivated Sepharose beads. The 6AP and GA affinity beads were very recently used and allowed to determine that protein folding activity of the rRNA (Ribosome-borne Protein Folding Activity, RPFA) is a selective target of both 6AP and GA (*31*). Here, using competition experiments on 6AP and GA affinity matrices with free quinacrine (QC) and chlorpromazine (CPZ), two other active antiprion compounds, we found that QC, but not CPZ, may also target the ribosome directly.

EXPERIMENTAL PROCEDURES

Chemical Methods. Reactions were monitored by TLC using Merk silica gel 60F-254 thin layer plates. Column chromatography experiments were carried out on SDS Chromagel 60 ACC, 40−63 μm. Column chromatography (CC) was performed using silica gel (70−200 μm). Melting points were determined on a Kofler hot-stage (Reichert) and are uncorrected. NMR spectra were recorded on Bruker Avance 400 MHz. Chemical shifts are given in parts per million (ppm) downfield of tetramethylsilane (TMS) used as an internal standard. Infrared spectra were recorded on a FTIR Schimadzu 8300. The HPLC analyses were carried out on a system consisting of a Waters 600 system controller, a Jones chromatography heater−chiller oven, and a Waters 994 photodiode array detector. Mass spectra (electrospray ionization (ESI) were measured on a TSQ Quantum (Thermo Electron Corporation) instrument. Elemental analyses for C, H, and N were performed on a Thermoquest EA-110 apparatus. 6AP was prepared as described previously (*32, 33*). Commercially available GA was used or obtained upon condensation of aminoguanidine with 2,6-dichlorobenzaldehyde.

(*R*)-6-(1-Hydroxybut-2-ylamino)phenanthridine, 6APi (1b). A mixture of 6-chlorophenanthridine (2.14 g, 10 mmol), (*R*)-2-aminobutanol (3 mL, 35 mmol), and NEt_3 (3 mL, 40 mmol) was heated at 110 °C for 2 h. After cooling to rt, 30 mL H_2O was added, and the mixture was extracted with CH_2Cl_2 (2 × 10 mL). The solvent was evaporated and **1b** crystallized from EtOAc. Mp 176−178 °C. Anal. ($C_{17}H_{18}N_2O$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 1.19 (t, J = 7 Hz, 3H, C*H*$_3$); 1.78 (p, 2H, CH_2CH$_3$); 3.76 (t, 1H, J = 9 Hz, 1H, CH_2OH); 3.98 (d, J = 10.9 Hz, 1H, CH_2OH); 4.26 (m, 1H, C*H*CH$_2$OH); 5.57 (br s, 1H, O*H*); 6.75 (br s, 1H, N*H*); 7.34 (t, $J_{1-2} = J_{2-3}$ = 7.5 Hz, 1H, 2-H); 7.54 (t, $J_{7-8} = J_{8-9}$ = 7.5 Hz, 1H, 8-H); 7.59 (t, 1H, 3-H); 7.70 (d, J_{3-4} = 8 Hz, 1H, 4-H); 7.77 (t, 1H, J_{9-10} = 7.5 Hz, 9-H); 7.86 (d, 1H, 7-H); 8.31 (d, 1H, 1-H); 8.51 (d, 1H, 10-H). ^{13}C NMR ($CDCl_3$) δ 10.8; 24.7; 56.8; 68.2; 118.5; 120.3; 121.5; 121.8; 122.5; 122.6; 125.7; 126.7; 128.7; 130.2; 133.6; 142.9; 153.7.

2-Chloro-6-fluoro-benzylideneaminoguanidine, GAi (2b). Aminoguanidine hydrochloride (2.20 g, 20 mmol) was added to a solution of 2-chloro-2-fluorobenzaldehyde (3.16 g, 20 mmol) in 50 mL EtOH followed by the addition of AcONa, $5H_2O$ (2.72 g, 20 mmol). After 2 h reflux, the hot mixture was filtered, and **2b** precipitated upon cooling to rt. Yield 87%. Mp 164−167 °C. Anal. ($C_8H_8ClFN_4$) C, H, N. ^{1}H NMR (DMSO) δ 5.50 (br s, 4H, 2 N*H* + N*H*$_2$); 7.15 (t, $J_{3-4} = J_{4-5}$ = 8 Hz, 1H, 4-H); 7.50 (d, 1H, 3-H); 7.65 (dd, J_{F-5} = 10 Hz, 1H, 5-H); 8.20 (s, 1H, NC*H*).

Benzyloxy-6-aminophenanthridines 1c−e (reaction in liquid ammonia). Ammonia (200 mL) was introduced into a flask connected with an efficient condenser followed by $Fe(NO_3)_3$ (0.02 g) and piece by piece Na (0.23 g, 10 mmol) additions. The solution was stirred for 0.5 h during this period; the initial blue color of the solution of Na gradually turned to gray indicating the complete conversion into $NaNH_2$. To this suspension, benzyloxyanilines (1.99, 10 mmol) dissolved in the minimum amount of anhydrous Et_2O were added. The mixture was stirred for 0.5 h followed by the addition of 2-chlorobenzonitrile (1.37 g, 10 mmol) in anhydrous Et_2O. The mixture was stirred for 2 h, and lithium (0.14 g, 20 mmol) was then added. After 2 h stirring, NH_4Cl (10 g) was gradually introduced under vigorous stirring. Ammonia was evaporated overnight. After addition of H_2O (50 mL), the mixture was extracted with CH_2Cl_2 (3 × 30 mL), and the organic layer was first washed with brine (40 mL), then dried with Na_2SO_4 and evaporated.

The residue was applied to a silica gel column and eluted first with CH_2Cl_2 and then with CH_2Cl_2−MeOH (99:1 to 95:5).

1-Benzyloxy-6-aminophenanthridine (1c) and 3-benzyloxy-6-aminophenanthridine (1e). 3-Benzyloxyaniline led to a mixture of compounds **1c** and **1e**. The two isomers could not be separated by column chromatography. They were isolated in 23% yield as a (1:1) mixture.

2-Benzyloxy-6-aminophenanthridine (1d). Prepared from 4-benzyloxyaniline. Yield 45%. Mp 138−141 °C. Anal. ($C_{20}H_{16}N_2O$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 5.22 (s, 2H, CH_2); 5.47 (brs, 2H, NH_2); 7.31 (dd, $J_{3-4} = 9$ Hz, $J_{1-3} = 2.4$ Hz, 1H, 3-H); 7.32 (t, $J = 7.2$ Hz, 1H, C$_6H_5$); 7.37 (t, 2H, $J = 7.2$ Hz, C$_6H_5$); 7.41 (m, 3H, C$_6H_5$ + 8-H); 7.52 (d, $J_{3-4} = 8$ Hz, 1H, 4-H); 7.69 (t, 1H, 9-H); 7.86 (s, 1H, 1-H); 8.09 (d, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 7-H); 8.42 (d, $J_{9-10} = 8.2$ Hz, 10-H).

3-Benzyloxy-6-aminophenanthridine (1e) (by Suzuki-Miyaura cross coupling). A solution of 5-benzyloxy-2-bromoaniline (1 g, 3.6 mmol) in 20 mL dioxane and 6 mL 1 M Na_2CO_3 was stirred under N_2 for 5 min. Pd[P(C$_6H_5$)$_3$]$_4$ (0.2 g, 0.17 mmol) was then introduced followed by 2-(4,4,5,5-tetramethyl-[1,3,2]-dioxaborolan-2-yl)-benzonitrile (3.6 mmol, 0.825 g). The mixture was refluxed for 12 h and then concentrated. The residue was extracted with CH_2Cl_2 (3 × 10 mL). The organic solution was washed with H_2O and dried with Na_2SO_4 and evaporated. The final compound was purified by column chromatography (CH_2Cl_2−EtOH, 98:2) and crystallized from EtOAc. Yield: 63%. Mp 121−123 °C. Anal. ($C_{20}H_{16}N_2O$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 5.20 (s, 2H, CH_2); 5.32 (br s, 2H, NH_2); 7.11 (dd, $J_{1-2} = 9.2$ Hz, $J_{2-4} = 2.4$ Hz, 1H, 2-H); 7.26 (d, 1H, 4-H); 7.34 (t, $J_{3'-4'} = J_{4'-5'} = 7.2$ Hz, 1H, 4′-H); 7.41 (t, 2H, $J_{2'-3'} = J_{5'-6'} = 7.2$ Hz, 3′-H and 5′-H); 7.50 (d, 2H, 2′-H, and 6′-H); 7.55 (td, $J_{8-10} = 1$ Hz, $J_{8-9} = 8$ Hz, 1H, 8-H); 7.76 (td, $J_{9-10} = 0.8$ Hz, 1H, 9-H); 7.86 (d, $J_{7-8} = 8$ Hz, 1H, 7-H); 8.34 (d, 1H, 1-H); 8.44 (d, $J_{9-10} = 8.4$ Hz, 1H, 10-H). ^{13}C NMR ($CDCl_3$) δ 70.5; 108.7; 114.6; 115.9; 117.9; 122.7; 123.4; 123.7; 126.4; 128.0; 128.4; 129.0; 131.2; 134.8; 137.3; 146.2; 155.5; 160.1. IR (KBr) γ 517; 621; 681; 718; 757; 811; 856; 890; 994; 1020; 1085; 1135; 1178; 1220; 1253; 1300; 1357; 1402; 1460; 1514; 1547; 1600; 1657; 1755; 1824; 1958; 2368; 2848; 2851; 3114; 3314; 3742.

Phthalylation of Benzyloxy-6-Aminophenanthridines. The amines **1c**−**e** (0.7 g, 2.3 mmol) were dissolved in THF (30 mL) containing 1 mL NEt_3. Phthalic anhydride (0.34 g, 2.3 mmol) was then added, and the mixture was refluxed for 6 h. THF was removed under reduced pressure. Water (20 mL) was added and the mixture extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was dried (Na_2SO_4) and evaporated. The residue was subjected to chromatography (CH_2Cl_2 100%) and crystallization from *i*-PrOH.

2-(1-Benzyloxyphenanthridin-6-yl)-isoindole-1,3-dione (3c). Yield: 80%. Mp 189−193 °C. Anal. ($C_{28}H_{18}N_2O_3$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 5.45 (s, 2H, CH_2); 7.33 (d, $J_{2-3} = 8$ Hz, 1H, 2-H); 7.42 (t, $J_{3'-4'} = J_{4'-5'} = 7.2$ Hz, 1H, 4′-H); 7.47 (t, $J_{2'-3'} = J_{5'-6'} = 7.2$ Hz, 2H, 3′-H and 5′-H); 7.60 (d, 2H, 2′-H and 6′-H); 7.65 (t, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 8-H); 7.70 (t, $J_{3-4} = 8$ Hz, 1H, 3-H); 7.80 (t, 1H, 9-H); 7.88 (m, 3H, phthalyl and 4-H); 7.93 (d, 1H, 7-H); 8.05 (dd, $J = 5.6$ Hz, $J = 3.2$ Hz, 2H, phthalyl); 9.72 (d, $J_{9-10} = 8.8$ Hz, 1H, 10-H). MS (E$^+$) *m*/*z*: 431 [M + H]$^+$; 453 [M + Na]$^+$.

2-(2-Benzyloxy-phenanthridin-6-yl)-isoindole-1,3-dione (3d). Yield: 81%. Mp 138−141 °C. Anal. ($C_{28}H_{18}N_2O_3$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 5.30 (s, 2H, CH_2); 7.40 (t, $J_{3'-4'} = J_{4'-5'} = 7.2$ Hz, 1H, 4′-H); 7.45 (t, $J_{2'-3'} = J_{5'-6'} = 7.2$ Hz, 2H, 3′-H + 5′-H); 7.50 (dd, $J_{3-4} = 9.4$ Hz, $J_{1-3} = 2.4$ Hz, 1H, 3-H); 7.57 (d, 2H, 2′-H and 6′-H); 7.69 (t, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 8-H); 7.90 (m, 4H, phthalyl, 9-H and 4-H); 8.05 (m, 3H, phthalyl and 1-H); 8.15 (d, 1H, 7-H); 8.62 (d, $J_{9-10} = 8.8$ Hz, 1H, 10-H).

2-(3-Benzyloxyphenanthridin-6-yl)-isoindole-1,3-dione (3e). Yield: 83%. Mp 121−123 °C. Anal. ($C_{28}H_{18}N_2O_3$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 5.24 (s, 2H, CH_2); 7.35 (t, $J_{3'-4'} = J_{4'-5'} = 7.2$ Hz, 1H, 4′-H); 7.41 (t, $J_{2'-3'} = J_{5'-6'} = 7.2$ Hz, 2-H, 3′-H + 5′-H); 7.48 (dd, $J_{1-2} = 9.4$ Hz, $J_{2-4} = 2.4$ Hz; 1H, 2-H); 7.50 (d, 2H, 2′-H and 6′-H); 7.61 (t, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 8-H); 7.70 (d, 1H, 4-H); 7.87 (dd, $J = 3.2$ Hz, $J = 5.6$ Hz, 2H, phthalyl); 7.88 (m, 2H, 7-H and 9-H); 8.04 (dd, 2H, phthalyl); 8.55 (d, 1H, 1-H); 8.61 (d, $J_{9-10} = 8.8$ Hz, 10-H). ^{13}C NMR ($CDCl_3$) δ 70.7; 111.4; 119.6; 120.4; 122.5; 123.3; 123.9; 124.5; 126.1; 127.4; 128.1; 128.6; 129.1; 132.1; 132.6; 135.1; 135.5; 136.7; 145.3; 146.4; 159.9; 167.7. IR (KBr) γ 537; 575; 631; 681; 700; 721; 755; 785; 814; 842; 884; 947; 1027; 1072; 1102; 1138; 1181; 1214; 1257; 1302; 1370; 1404; 1452; 1472; 1574; 1600; 1715; 1792; 2991; 3031; 3044; 3434; 3600; 3708; 3831; 3894 cm^{-1}. MS (E+) *m*/*z*: 431 [M + H]$^+$; 453 [M + Na]$^+$.

Debenzylation of 2-(Benzyloxyphenanthridin-6-yl)-isoindole-1,3-diones. Preparation of Phenols 4c−e. 0.1 g of 10% Pd/C and 0.5 mL of AcOH were added to a solution of 2-(benzyloxyphenanthridin-6-yl)-isoindole-1,3-dione (1.9 mmol, 0.65 g) in 50 mL EtOH and 50 mL CH_2Cl_2. The mixture was stirred under H_2 atmosphere (AP) for 12 h. The catalyst was removed by filtration through a Whatman filter. The filter was washed once with 5 mL EtOH. The phenols crystallized upon concentration of the ethanolic solution.

2-(1-Hydroxyphenanthridin-6-yl)-isoindole-1,3-dione (4c). Yield: 97%. Mp 189−193 °C. Anal. ($C_{21}H_{12}N_2O_3$) C, H, N.^{1}H NMR ($CDCl_3$) δ 7.23 (d, $J_{2-3} = J_{3-4} = 8$ Hz, 1H, 2-H); 7.52 (t, 1H, 3-H); 7.59 (t, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 8-H); 7.68 (d, 1H, 4-H); 7.82 (m, 4H, phthalyl and 7-H and 9-H); 7.97 (dd, $J = 3.2$ Hz, $J = 5.6$ Hz, phthalyl); 9.80 (d, $J_{9-10} = 8.4$ Hz, 1H, 10-H); 10.9 (br s, 1H, OH).

2-(2-Hydroxyphenanthridin-6-yl)-isoindole-1,3-dione (4d). Yield: 82%, Mp 201−203 °C. Anal. ($C_{21}H_{12}N_2O_3$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 7.35 (t, 1H); 7.63 (dd, 1H, $J_{3-4} = 8.5$ Hz, $J_{1-3} = 3$ Hz, 3-H); 7.71 (t, 1H); 7.95 (m, 5H, phthalyl + 1H); 8.10 (m, 4H, phthalyl); 8.18 (d, 1H, 7-H); 8.60 (d, 1H, 10-H); 8.75 (brs, OH).

2-(3-Hydroxyphenanthridin-6-yl)-isoindole-1,3-dione (4e). Yield: 98%. Mp 169−172 °C. Anal. ($C_{21}H_{12}N_2O_3$) C, H, N.^{1}H NMR ($CDCl_3$) δ 7.30 (dd, $J_{1-2} = 8.8$ Hz, $J_{2-4} = 2.4$ Hz, 1H, 2-H); 7.49 (t, $J_{7-8} = J_{8-9} = 8$ Hz, 1H, 8-H); 7.52 (d, 1H, 4-H); 7.80 (m, 4H, phthalyl + 7-H + 9-H); 7.97 (dd, $J = 5.6$ Hz, $J = 3.2$ Hz, phthalyl); 8.41 (d, 1H, 1-H); 8.52 (d, $J_{9-10} = 8.2$ Hz, 1H, 10-H); 9.50 (brs, 1H, OH).

Preparation of Spacers (5a,b). *N*-Hydroxybenzotriazole (2.83 g, 21 mmol) and dicyclohexylcarbodiimide (4.14 g, 20 mmol) were added to a cooled (5 °C) solution of *ε*-*Z*-aminocaproic acid (6.62 g, 25 mmol) in 50 mL EtOAc. The mixture was left under stirring for 2 h at rt. The precipitated dicyclohexylurea was filtrated and washed once with 10 mL EtOAc. 5-Aminopentan-1-ol (2.16 g, 21 mmol) and NEt_3 (5 mL) were added to the combined filtrates. After 3 h stirring, the mixture was diluted in 150 mL EtOAc and washed with 20 mL 1 N HCl followed by 50 mL saturated $NaHCO_3$ and then with 20 mL H_2O. Derivatives **5** crystallized upon concentration of the solvent. Further purification could be achieved by crystallization from EtOAc.

5-(6-Benzyloxycarbonylaminohexanoylamino)pentan-1-ol (5a). Yield: 85%. Mp: 108−110 °C. Anal. ($C_{19}H_{30}N_2O_4$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 1.25−1.85 (m, 12H, 6 × CH_2); 2.15 (t, $J = 7.4$ Hz, 2H, CH_2CO); 3.19 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.26 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.64 (t, $J = 6.4$ Hz, CH_2OH); 4.88 (br s, 1H, NH); 5.08 (s, 2H, CH_2C$_6H_5$); 5.56 (br s, 1H, N*H*); 7.28−7.38 (m, 5H, C$_6H_5$). ^{13}C NMR ($CDCl_3$) δ 23.4; 25.3; 25.6; 25.9; 29.7; 30.0; 32.5; 36.9; 39.6; 41.2; 62.9; 67.0; 128.5; 128.9; 137.0; 156.9; 173.3. IR (KBr) γ 528; 586; 700; 636;

779; 950; 986; 1042; 1200; 1236; 1214; 1235; 1258; 1329; 1434; 1474; 1528; 1621; 1679; 2857; 2871; 3015; 3331; 3365; 3378; 3600; 3729 cm^{-1}. MS (E^+) *m/z*: 351 $[M + H]^+$; 373 $[M + Na]^+$.

5-(6-*tert*-Butyloxycarbonylaminohexanoylamino)pentan-1-ol (5b). Yield 67%. Mp 67−72 °C. Anal. ($C_{15}H_{30}N_2O_4$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.3 (m, 12 H, 6 × CH_2); 1.5 (s, 9H, $(CH_3)_3$); 2.1 (t, 2H, CH_2CO); 3.03 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.20 (q, 2H, CH_2NH); 3.55 (t, 2H, CH_2OH); 4.55 (s, 1H, NHCOO); 5.5 (s, 1H, CH_2NHCO). MS (E^+) *m/z*: 303$[M + H]^+$; 325 $[M + Na]^+$.

General Procedure for the Mitsunobu Reaction. Synthesis of Conjugates (6c−e). *Z*-Aminocaproylaminopentanol (**5a**) (1.8 mmol, 0.63 g) and triphenylphosphine (1.8 mmol, 0.47 g) were added to a solution of 2-(hydroxyphenanthridin-6-yl)-isoindole-1,3-dione (**4c−e**) (1.8 mmol, 0.61 g) in THF (30 mL). The mixture was cooled to 0 °C, and *iso*-propyl diazodicarboxylate (1.8 mol, 0.378 g) was introduced under vigorous stirring. After 2 h at rt, the reaction mixture was concentrated and H_2O (20 mL) was added. The aqueous suspension was extracted with CH_2Cl_2 (3 × 15 mL), the organic layer was dried with Na_2SO_4, and the solvent was evaporated. The final compound was obtained after column chromatography (EtOAc−CH_2Cl_2−toluene: 50−25−25).

(5-{5-[6-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-phenanthridin-1-yloxy]-pentylcarbamoyl}-pentyl)-carbamic Acid Benzyl Ester 6c. Yield 92%. Mp 134−137 °C. Anal. (C_{40} $H_{40}N_4O_6$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.58 to 1.68 (m, 12H, (6 × CH_2); 2.09 (t, $J = 7.4$ Hz, 2H, CH_2CO); 3.14 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.32 (q, $J = 6.4$ Hz, 2H, CH_2NH); 4.32 (t, $J = 6.4$ Hz, 2H, CH_2O); 4.83 (br s, 1H, NH); 5.07 (s, 2H, $CH_2C_6H_5$); 5.46 (br s, 1H, NH); 7.24 (d, $J_{2-3} = J_{3-4} = 8$ Hz, 1H, 2-H); 7.34 (m, 5H, C_6H_5); 7.68 (m, 2H, 3-H + 8-H); 7.82 (m, 4H, phthalyl + 9-H + 4-H); 7.89 (d, $J_{7-8} = 8$ Hz, 1H, 7-H); 8.02 (dd, $J = 5.6$ Hz, $J = 3.2$ Hz, phthalyl); 9.71 (d, $J_{9-10} = 8.4$ Hz, 1H, 10-H). ^{13}C NMR ($CDCl_3$) δ 24.0; 25.4; 26.5; 29.2; 29.7; 29.8; 36.8; 39.6; 41.0; 66.8; 69.3; 110.4; 115.9; 123.3; 124.3; 124.4; 125.4; 127.6; 128.3; 128.5; 128.8; 129.0; 131.7; 132.4; 134.9; 135.3; 136.9; 145.4; 146.4; 156.7; 157.7; 167.6; 173.1. IR (KBr) cm^{-1} 478; 532; 671; 717; 771; 887; 964; 1033; 1080; 1157; 1273; 1373; 1458; 1535; 1635; 1689; 1728; 1913; 2314; 2862; 2931; 3063; 3302; 3749; 3865. MS (E^+) *m/z*: 673 $[M + H]^+$; 695 $[M + Na]^+$.

(5-{5-[6-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-phenanthridin-2-yloxy]-pentylcarbamoyl}-pentyl)-carbamic Acid Benzyl Ester (6d). Yield 65%. Mp 131−132 °C. Anal. (C_{40} $H_{40}N_4O_6$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.50 to 1.52 (m, 12H, 6 × CH_2); 2.18 (t, $J = 7.4$ Hz, 2H, CH_2CO); 3.20 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.25 (q, $J = 6.4$ Hz, 2H, CH_2NH); 4.22 (t, $J = 6.4$ Hz, 2H, CH_2O); 4.80 (brs, 1H, NH); 5.10 (s, 2H, $CH_2C_6H_5$); 5.50 (brs, 1H, NH); 7.37 to 7.48 (m, 5H, C_6H_5); 7.54 (d, $J_{3-4} = 8$ Hz, 1H, 3-H); 7.68 (m, 2H, 4-H and 8-H); 7.89 (m, 3H, phthalyl and 9-H); 7.95 (s, 1H, 1-H); 8.45 (dd, $J = 3.2$ Hz, $J = 5.6$ Hz, phthalyl); 8.12 (d, $J_{7-8} = 8$ Hz, 1H, 7-H); 8.64 (d, $J_{9-10} = 8.2$ Hz, 1H, 10-H). MS (E^+) *m/z*: 673 $[M + H]^+$; 695 $[M + Na]^+$.

(5-{5-[6-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)-phenanthridin-3-yloxy]-pentylcarbamoyl}-pentyl)-carbamic Acid Benzyl Ester (6e). Yield 97%. Mp 155−157 °C. Anal. (C_{40} $H_{40}N_4O_6$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.41−1.67 (m, 12H, 6 CH_2); 2.12 (t, $J = 7.4$ Hz, 2H, CH_2CO); 3.11 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.19 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.24 (t, $J = 6.4$ Hz, 2H, CH_2OAr); 4.81 (br s, 1H, NH); 5.01 (s, 2H, $CH_2C_6H_5$); 5.71 (brs, 1H, NH); 7.24 (m, 5H, C_6H_5); 7.32 (dd, $J_{1-2} = 8$ Hz, $J_{2-3} = 2.4$ Hz, 2-H); 7.55 (m, 2H, 4-H and 8-H); 7.81 (m, 4H, phthalyl and 7-H and 9-H); 7.98 (dd, $J = 3.2$ Hz, $J = 5.6$ Hz, phthalyl); 8.48 (d, 1H, 1-H); 8.55 (d, $J_{9-10} = 8.4$ Hz, 10-H). MS (E^+) *m/z*: 673 $[M + H]^+$; 695 $[M + Na]^+$.

Removal of the Phthalyl Protecting Group: Preparation of 7c−e. Hydrazine hydrate (0.2 mL) was added to a solution of **6c−e** (0.336 g, 0.5 mmoL) in DMF (1 mL). The mixture was heated at 70 °C for 15 min and then cooled to rt. After evaporation under vacuum, the solid residue was taken up with CH_2Cl_2 (30 mL). The solution was washed with H_2O (2 × 5 mL), dried, and concentrated. Compounds **7c−e** were purified by column chromatography (CH_2Cl_2−EtOH−NEt_3, 95:5:0.1).

6-Amino-1-(benzyloxycarbonylaminohexanoylaminopentyloxy)phenanthridine (7c). Yield 65%. Mp 147−151 °C. Anal. ($C_{32}H_{38}N_4O_4$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.51 to 1.72 (m, 12H, (6 × CH_2); 2.05 (t, $J = 7.0$ Hz, 2H, CH_2CO); 3.20 (q, $J = 6.5$ Hz, 2H, CH_2NH); 3.38 (q, $J = 6.5$ Hz, 2H, CH_2NH); 4.23 (t, $J = 6.5$ Hz, 2H, CH_2O); 5.12 (s, 2H, $CH_2C_6H_5$); 5.55 (br s, 3H, NH); 7.25 (d, $J_{2-3} = J_{3-4} = 8$ Hz, 1H, 2-H); 7.24 (m, 5H, C_6H_5); 7.73 (m, 2H, 3-H and 8-H); 7.82 (m, 2H, 9-H and 4-H); 7.85 (d, $J_{7-8} = 8$ Hz, 1H, 7-H); 9.45 (d, $J_{9-10} = 8.4$ Hz, 1H, 10-H). MS (E+) *m/z*: 543 $[M + H]^+$; 565 $[M + Na]^+$.

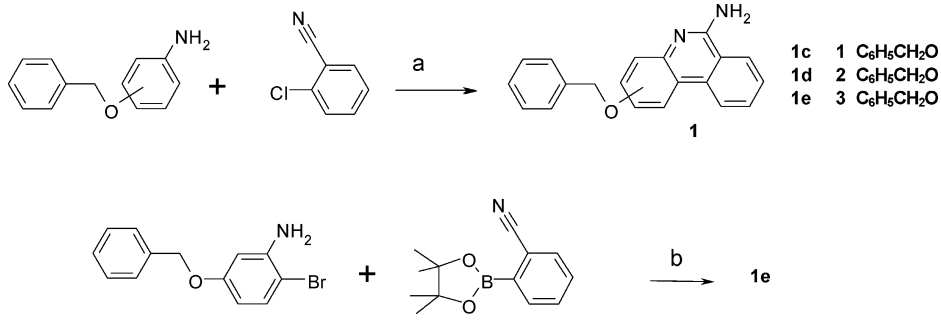
6-Amino-2-(benzyloxycarbonylaminohexanoylaminopentyloxy)phenanthridine (7d). Yield 62%, Mp 118−122 °C. Anal. ($C_{32}H_{38}N_4O_4$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.47 to 1.62 (m, 12H, 6 × CH_2); 2.23 (t, $J = 6.5$ Hz, 2H, CH_2CO); 3.20 (q, $J = 6.5$ Hz, 2H, CH_2NH); 3.35 (q, $J = 6.5$ Hz, 2H, CH_2NH); 4.22 (t, $J = 6.4$ Hz, 2H, CH_2O); 4.85 (brs, 1H, NH); 5.12 (s, 2H, $CH_2C_6H_5$); 5.85 (br s, 2H, NH_2); 7.32 (m, 5H, C_6H_5); 7.52 (d, $J_{3-4} = 7.5$ Hz, 1H, 3-H); 7.65 (m, 2H, 4-H + 8-H); 7.85 (m, 1H, 9-H); 7.96 (s, 1H, 1-H); 8.10 (d, $J_{7-8} = 8$ Hz, 1H, 7-H); 8.54 (d, $J_{9-10} = 8.2$ Hz, 1H, 10-H).

6-Amino-3-(benzyloxycarbonylaminohexanoylaminopentyloxy)phenanthridine (7e). Yield 48%. Mp: 123−125 °C. Anal. ($C_{32}H_{38}N_4O_4$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.42−1.69 (m, 12H, 6 CH_2); 2.15 (t, $J = 7.3$ Hz, 2H, CH_2CO); 3.13 (q, $J = 6.4$ Hz, 2H, CH_2NH); 3.22 (q, $J = 6.4$ Hz, 2H, CH_2NH); 4.06 (t, $J = 6.6$ Hz, 2H, CH_2OAr); 5.01 (s, 2H, $CH_2C_6H_5$); 5.71 (br s, 2H, NH_2); 7.24 (m, 5H, C_6H_5); 7.39 (dd, $J_{1-2} = 8$ Hz, $J_{2-3} = 2.4$ Hz, 2-H); 7.52 (m, 2H, 4-H and 8-H); 7.81 (m, 2H, 7-H + 9-H); 8.38 (d, 1H, 1-H); 8.50 (d, $J_{9-10} = 8.2$ Hz, 10-H). MS (E^+) *m/z*: 543 $[M + H]^+$; 565 $[M + Na]^+$.

Removal of the Z-group. Preparation of 3-(6-Aminocaproylaminopentyloxy)-6-phtalimidophenanthridine (8e). Pd/C 10% (0.1 g) and 35% hydrochloric acid (0.2 mL) were added to a solution of **6e** (0.336 g, 0.5 mmoL) in 10 mL EtOH and 10 mL CH_2Cl_2. The mixture was stirred under H_2 atmosphere (AP) for 12 h. The catalyst was removed by filtration through a Whatman filter. The filter was rinsed once with 1 mL EtOH. The hydrochloride was isolated upon concentration under vacuum and used in the immobilization step without further purification.

{5-[5-(3,5-Dichloro-4-formylphenyloxy)-pentylcarbamoyl]-pentyl}-carbamic Acid *tert*-Butyl Ester (11). Boc-aminocaproylaminopentanol (3 mmol, 0.906 g) and triphenylphosphine (3 mmol, 0.786 g) were added to a solution of 2,6-dichloro-4-hydroxybenzaldehyde **10** (*35*) (3 mmol, 0.525 g) dissolved in 30 mL of THF. The mixture was cooled to 0 °C, and *iso*-propyl diazodicarboxylate (3 mmol, 0.590 g) was introduced under vigorous stirring. After 2 h at rt, the reaction mixture was concentrated and H_2O (20 mL) was added. The aqueous suspension was extracted with CH_2Cl_2 (3 × 10 mL), and the organic layer was dried with Na_2SO_4 and the solvent evaporated. The final compound was obtained after column chromatography (EtOAc−toluene: 50−50). Yield: 83%. Mp: 68−70 °C. Anal. ($C_{23}H_{34}Cl_2N_2O_5$) C, H, N. ^1H NMR ($CDCl_3$) δ 1.52 (s, 9H, $C(CH_3)_3$); 1.36−1.71 (m, 10H, CH_2); 1.86 (q, 2H, CH_2); 2.20 (t, 2H, $J = 7.5$ Hz, CH_2CO); 3.12 (dd, 2H, $J = 6.3$ Hz, CH_2NH); 3.29 (dd, 2H, $J = 6.3$ Hz, CH_2NH); 4.04 (t, 2H, $J = 6.1$ Hz,

Scheme 1. Preparation of Benzyloxy-6-aminophenanthridines 1c−e[a]



[a] (a) $NaNH_2$, $LiNH_2$, NH_3; (b) Na_2CO_3, $Pd[P(C_6H_5)_3]$, dioxane, H_2O.

CH_2O); 4.56 (br s, 1H, NH); 5.54 (br s, 1H, NH); 6.92 (s, 2H, H-3 + H-5); 10.44 (s, 1H, C*H*O).

{5-[5-(3,5-Dichloro-4-benzylidenaminoguanidine)-pentylcarbamoyl]-pentyl}-carbamic Acid *tert*-Butyl Ester (12). Aminoguanidine hydrochloride (0.22 g, 2 mmol) was added to a solution of conjugate **11** (0.98 g, 2 mmol) in 10 mL EtOH followed by the addition of AcONa, $5H_2O$ (0.27 g, 2 mmol). After 2 h refluxing, the hot mixture was filtered, and **12** precipitated upon cooling to rt. Yield: 54%. Anal. ($C_{24}H_{38}Cl_2N_6O_4$) C, H, N. ^{1}H NMR ($CDCl_3$) δ 1.24−1.63 (m, 10H, 5 × CH_2); 1.43 (s, 9H, $C(CH_3)_3$); 1.74 (q, 2H, CH_2); 2.17 (t, J = 7 Hz, 2H, CH_2CO); 3.08 (q, J = 6.03 Hz, 2H, CH_2NH); 3.25 (q, 2H, CH_2NH); 3.95 (t, J = 6 Hz, 2H, CH_2−O); 5.79 (br s, 2H, NH_2); 6.35 (br s, 1H, NH); 6.88 (s, 2H, 3-H and 5-H); 8.36 (s, 1H, NCH). ^{13}C NMR ($CDCl_3$) δ 23.0; 25.1; 26.1; 28.2; 28.3; 29.0; 29.4; 36.1; 39.0; 68.0; 78.6; 115.2; 123.9; 134.8; 141.4; 155.9; 157.9; 160.6; 172.9.

2,6-Dichloro-4-(ε-aminocaproylaminopentyloxy)-benzylidenaminoguanidine) Trifluoroacetate (13). CF_3COOH (1 mL) was added to a solution of **12** (0.27 g, 0.5 mmol) in CH_2Cl_2 (10 mL). The solution was stirred for 2 h at rt and then concentrated until dryness. The viscous oil crystallized upon trituration with 2 mL cyclohexane followed by trituration with 2 mL Et_2O. Yield 76%, mp 75−78 °C. ^{1}H NMR (DMSO-d_6) δ 1.21−1.88 (m, 12 H, 6 × CH_2); 2.21 (t, J = 7 Hz, 2H, CH_2CO); 3.03 (m, 4H, 2CH_2NH); 4.02 (t, J = 6 Hz, 2H, CH_2−O); 6.23 (m, 4H, NH); 6.84 (s, 2H, 3-H and 5-H); 8.25 (s, 1H, NCH).

Immobilization of Ligands on Activated Sepharose Beads. Ligands were attached to CNBr-activated Sepharose 4 fast flow from Amersham Pharmacia according to the manufacturer's directions. A solution of 0.1 mmol ligands **8e** or **13** in 2 mL DMSO was added to 38 mL pH 8.3 solution (0.02 M $NaHCO_3$ + 0.1 M NaCl). This solution (0, 1, 2, 5 mL) was added to approximately 1 mL of beads suspended in pH 8.3 solution (final volume in each cartridge 10 mL). The cartridges were allowed to shake for 12 h at rt. The solutions were then drained from the beads, and 10 mL ethanolamine buffered solution (pH 8.5) was added to each cartridge, which was allowed to shake for 3 h at rt. This quenching step was repeated once. The phthalyl protecting group of 6AP-beads was then removed. A 3 mL solution of H_2O, DMF, and hydrazine hydrate (1:1:1) was added to the cartridges. After 15 min shaking, the solution was drained and the beads washed 3 times with 10 mL buffer. Finally, 10 mL buffer containing 1 g·L^{-1} NaN_3 was added to each cartridge, which was stored at 4 °C.

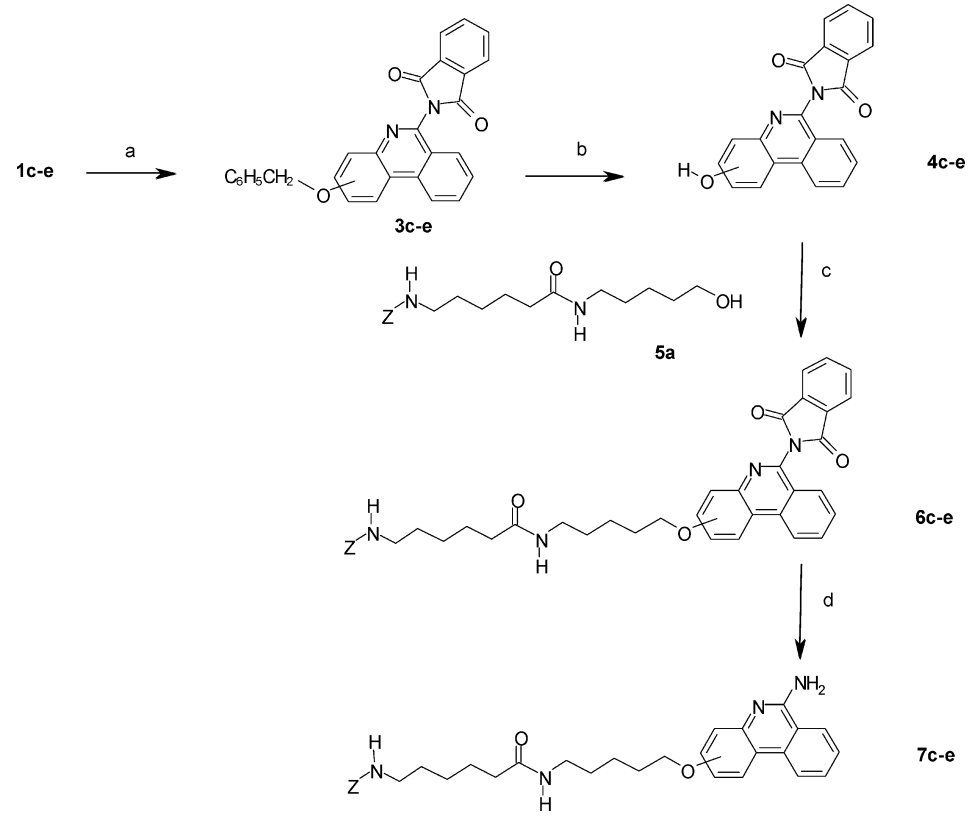
Affinity Chromatography Experiments on Immobilized 6AP and GA. These experiments were performed as previously described (*31*). Briefly, yeast (*Saccharomyces cerevisiae*) cell extracts were prepared using homogenization buffer (25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.2% Triton X-100, and 1 mM PMSF). Yeast protein extracts were obtained from a culture growing at 30 °C ($OD_{600\ nm}$ = 0.8). Cell pellets were resuspended in homogenization buffer (300 μL/100 mL of culture) and lysed using acid-washed glass beads (purchased from Sigma, 300 μL/100 mL of culture). Homogenates were vortexed for 30 s followed by 30 s ice-cooling (six times) and then centrifuged for 3 min at 3000 rpm at 4 °C. Supernatants were recovered, assayed for protein content (using Bio-Rad protein assay), and immediately loaded batchwise on the affinity matrices. Just before use, 6AP and GA beads were washed with 1 mL of bead buffer (50 mM Tris−HCl (pH 7.4), 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10 μg/mL of leupeptin, aprotinin, and soybean trypsin inhibitor, and 100 μM benzamidine) and diluted 4 times in this buffer. 200 μg of yeast protein extract were added to 40 μL of diluted beads (10 μL of packed beads). The volume was adjusted to 600 μL by adding bead buffer, and the tubes were rotated at 4 °C for 30 min. Competitions with active or inactive drugs were performed with free compounds (or the corresponding volume of DMSO) at a final concentration of 1 mM incubated with cell extracts for 3 min before the mixture was added to the affinity matrix. Then, bead buffer containing the same quantity (1 mM) of the corresponding free compound was added immediately (as described previously) to reach a final volume of 600 μL. After incubation, a brief spin at 10 000 *g* at 4 °C, and removal of the supernatant, the beads were washed 4 times with bead buffer before addition of 35 μL of 2× Laemmli sample buffer. Following heat denaturation for 3 min at 95 °C, bound proteins were analyzed by 10% SDS-PAGE as described below.

Electrophoresis and Silver Staining. The proteins bound to 6AP or GA matrices were separated by 10% SDS-PAGE (precast NuPAGE−InVitrogen−1 mm thick gel) followed by silver staining using a GE Healthcare Life Sciences SDS-PAGE silver staining kit.

RESULTS

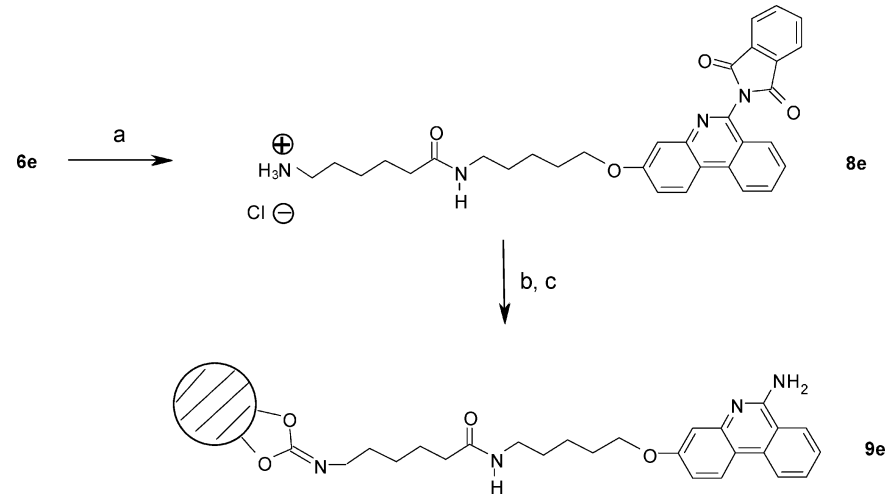
Preparation of 6-Aminophenanthridine Beads. The synthesis of the conjugates of 6AP is depicted in Schemes 1 and 2. Condensation of 2- or 3-benzyloxyaniline with 2-chlorobenzonitrile in liquid ammonia using metal amides (*32*) led to benzyloxy-6-aminophenanthridine **1c−e**. The mixture of **1c** and **1e** could not be separated on column chromatography. Alternatively, 3-benzyloxy-6-aminophenanthridine **1e** was obtained in a Suzuki−Myaura coupling procedure (*33*). Phthalylation of 6-aminophenanthridines **1c−e** afforded derivatives **3c−e**. The benzyl group was removed by catalytic hydrogenation to afford phenols **4c−e**. The spacer arms **5** were obtained by acylation of 5-aminopentanol with Boc or *Z*-ε-aminocaproic acid. Condensation of phenols **4c−e** with spacer **5a** under Mitsunobu reaction conditions led to conjugates **6c−e**. Finally, the phtalimido protecting group was eliminated to afford **8c−e**. Following the evaluation of **8c−e** in the antiprion test, the removal of

Scheme 2. Synthesis of 6AP Conjugates[a]



[a] (a) Phthalic anhydride, NEt_3, THF; (b) H_2, Pd/C; (c) $(C_6H_5)_3P$, di-*iso*-propyl diazodicarboxylate, THF; (d) hydrazine hydrate, H_2O, DMF.

Scheme 3. Synthesis of 6AP Beads[a]



[a] (a) H_2 Pd/C, HCl, EtOH; (b) Sepharose preactivated beads; (c) hydrazine hydrate, H_2O, DMF.

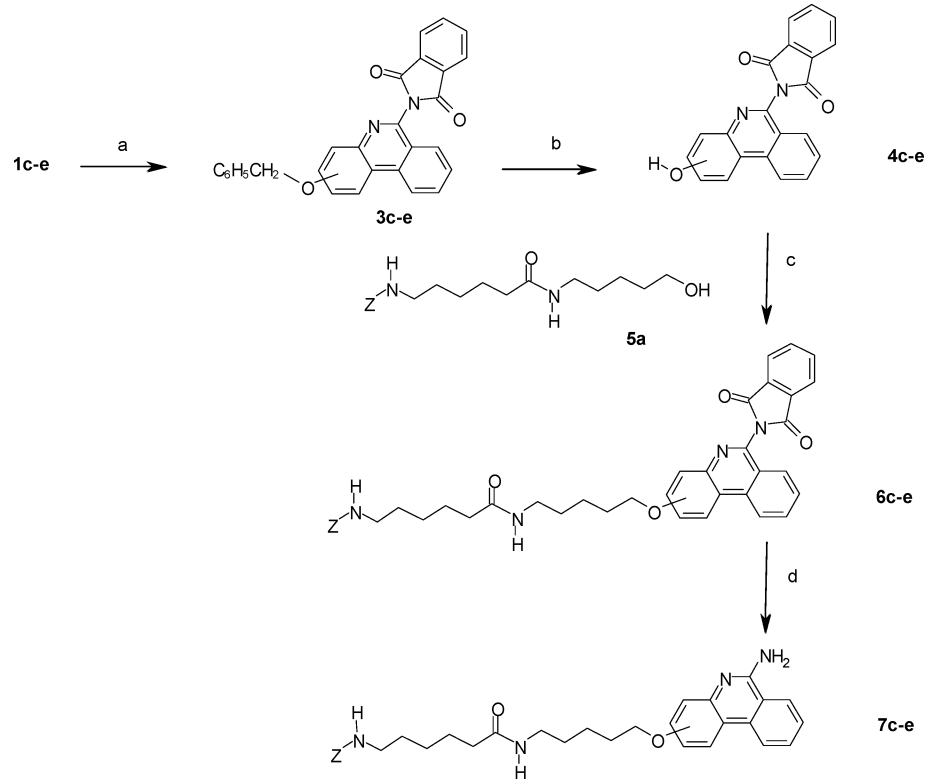
the Z-group was performed only on **6e**, which bears the spacer arm in position 3.

Elimination of the Z protecting group of **6e** led to the conjugate **8e**. The immobilization of conjugate **8e** was performed on commercially available preactivated beads (CNBr). Several ratios of ligand conjugated to the Sepharose beads were tested. In the following affinity chromatography experiments, optimal results were obtained with the matrices made using the bigger volume of ligand solution (5 mL). Residual activated groups were quenched by aminoethanol. Finally, the phthalyl protective group was removed by hydrazinolysis. Optimization of reaction conditions led to the selection of a DMF, H_2O, hydrazine hydrate (1:1:1) mixture (Scheme 3).

Preparation of Guanabenz Beads. The conjugate was easily obtained from 2,6-dichloro-4-hydroxybenzaldehyde **10** (*35*). Coupling of the Boc-protected spacer arm **5b** was achieved under Mitsunobu conditions leading to conjugate **11**. This aldehyde gave the protected GA conjugate **12** upon refluxing with aminoguanidine in EtOH. Acidic cleavage of the Boc protecting group led to the trifluoroacetate **13**, which was eventually coupled to Sepharose preactivated beads to obtain GA-beads **14** (Scheme 4).

Affinity Chromatography Assays. The ability of CPZ and QC to compete for ribosomal components binding on 6AP and GA beads is depicted in Figure 2. As can be seen, QC (lanes

Scheme 4. Preparation of GA Beads[a]



[a] (a) $(C_6H_5)_3P$, di-*iso*-propyl diazodicarboxylate, THF; (b) aminoguanidine, EtOH; (c) CF_3COOH, CH_2Cl_2; (d) Sepharose preactivated beads.

6), but not CPZ (lanes 5), was able to compete for the binding of ribosomal components to both 6AP and GA beads.

DISCUSSION

We have previously described two single-step routes for the synthesis of 6APs (*32, 33*). In the present work, these two approaches were used. In preliminary structure−activity studies, we noticed that derivatives substituted on the amino group were devoid of any antiprion activity. Therefore, **1b** was selected as an inactive control useful for the affinity chromatography assays. This also indicates that the spacer arm should not be linked to the 6-amino group. Further, this amino group of 6AP appeared to be rather nucleophilic and then, if unprotected, would likely react in the coupling procedure on the activated Sepharose beads. In order to circumvent this major problem, a protective group which could be eliminated after grafting the conjugate to the beads was required. It implies that the deprotection of the 6-amino group of 6AP derivatives should be achieved under mild conditions. The phthalyl protective group fulfilled this requirement. The antiprion activity of conjugates **7c**−**e** has been evaluated in the yeast prion-based assay. Only the **7e** derivative substituted in position 3 retained some antiprion activity of **1a**, whereas conjugates **7c** and **7d**, substituted in position 1 or 2 respectively, were totally inactive (*31*). Therefore, **8e** was selected for the affinity chromatography study. Concerning GA beads, we noticed that arginine-containing peptides have been previously grafted on Sepharose beads. In these assays, the unprotected amidine group did not interfere in the coupling process (*34*). Therefore, in contrast to the preparation of 6AP beads, it was estimated that the amidine group of Guanabenz conjugate did not need to be protected.

Affinity chromatography experiments were performed with both 6AP and GA beads. Interestingly, although the two compounds are chemically unrelated, they were both found to interact with the ribosome in an RNA-dependent manner (*31*). First, both 6AP and GA showed specific interactions with ribosomal components: most of the proteins specifically bound to 6AP or GA beads turned out to be ribosomal proteins. The binding of these ribosomal proteins was specific, as they were absent from control beads (Figure 2, panel B, lane 1 in both gels), present on 6AP or GA beads (lane 2) and respectively competed away by free 6AP (left gel, lane 3) or by free GA (right gel, lane 3) but not by free 6APi (left gel, lane 4) or by free GAi (right gel, lane 4). The interaction of both 6AP and GA with ribosomal components was then shown to be RNA-dependent, as a treatment of the cellular extracts with RNase A before affinity chromatography purification completely abolished the binding of all ribosomal proteins. 6AP and GA were further shown to selectively interact with the domain V of the large rRNA (rRNA) of the large subunit of the ribosome. Therefore, the binding of ribosomal components to 6AP or GA beads is best explained by postulating that, in affinity chromatography experiments, immobilized 6AP or GA retains the entire ribosome due to the ability to specifically interact with domain V of the large rRNA. Interestingly, in parallel to their ability to specifically interact with domain V of the large rRNA, both 6AP and GA were shown to specifically inhibit its protein folding activity. Indeed, in addition, to bear the peptidyl transferase activity involved in translation, domain V of the large rRNA of the large subunit of the ribosome has also been shown to be able to assist protein folding, at least *in vitro* (*36*). We thus found that both 6AP and GA inhibit this ribosome-borne protein folding activity (RPFA) without any effect on the peptidyl transferase activity of the ribosome. Both 6APi (**1b**) and GAi (**2b**), which are inactive as antiprion chemical derivatives of, respectively, 6AP and GA, were unable to compete for the binding of ribosomal components to both 6AP and GA beads and also to inhibit RPFA (*30*). We thus proposed that the antiprion effect of both 6AP and GA could be due to their ability to inhibit the RPFA, and thus, RPFA could be involved in prion propagation. Therefore,

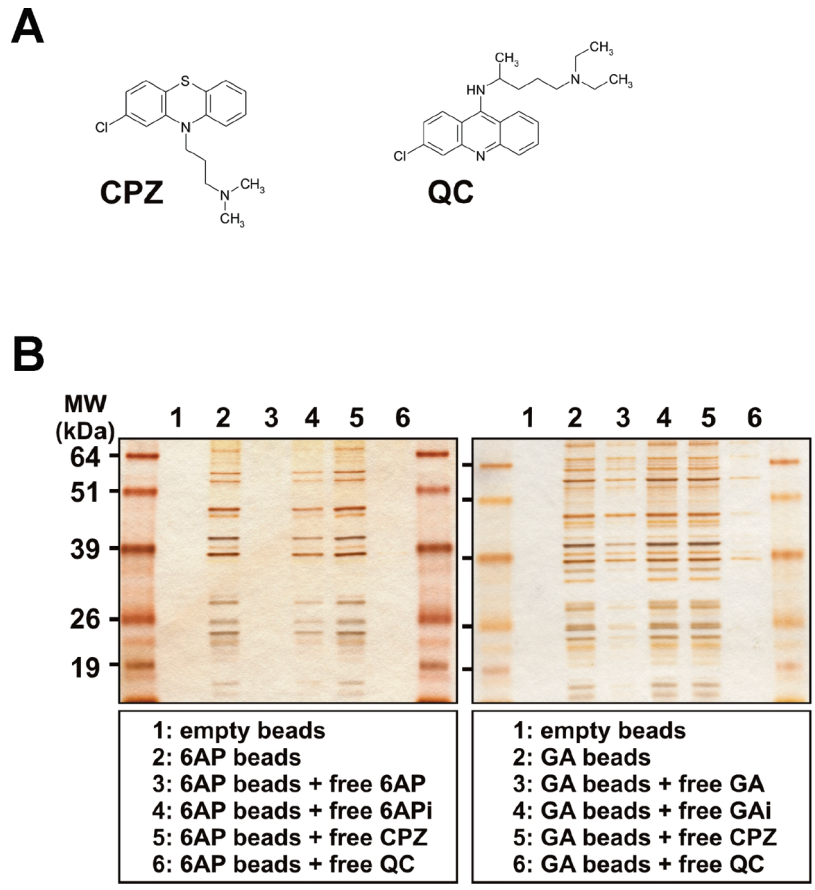


Figure 2. Competition experiments with Chlorpromazine (CPZ) and Quinacrine (QC). A. Molecular structures of CPZ and QC were depicted. B. Extracts from yeast were incubated with 6AP beads (left panel) or GA beads (right panel). The beads were then washed extensively and the bound proteins analyzed by SDS-PAGE. Proteins were then silver-stained. Most of the bands were previously identified as ribosomal proteins from both the small and large subunits of the ribosome. In both gels, lane 1 corresponds to chromatography using control beads without 6AP (left gel) or GA (right gel), and lane 2 to chromatography using 6AP (left gel) or GA (right gel) beads. Note that, in both cases, the patterns of eluted ribosomal proteins were rather similar. In both gels, lanes 3 to 6 correspond to competition experiments with the indicated free compounds. Note that QC, but not CPZ, was able to compete for the binding of ribosomal components to both 6AP and GA beads.

we were interested in determining if other known antiprion drugs could compete for the binding of ribosomal components on 6AP and GA beads when added to a mix of 6AP or GA beads and cellular extracts. This possibility is particularly appealing, as free 6AP was shown to efficiently compete for the binding of ribosomal components to GA beads, and vice versa (*31*), clearly indicating that they share overlapping binding sites on rRNA, which was indeed recently shown to be the case (*37*). Therefore, the ability of other unrelated antiprion drugs to compete for the binding of the ribosome to 6AP and GA beads would be an indication that these compounds share at least some common binding sites on domain V of the large rRNA of the large ribosomal subunit with 6AP or GA. Hence, all these molecules could share the same biological target accounting for their antiprion effect. Quinacrine (QC) and Chlorpromazine (CPZ) are two drugs already in use in humans for the treatment of malaria and psychosis, respectively. They were recently isolated as active *ex vivo* against mammalian prion in a N2a cell-based model (*8*) and the latter against yeast prions (*13*). The ability of CPZ and QC to compete for ribosomal components binding on 6AP and GA beads was determined (Figure 2). Interestingly, whereas QC was clearly able to efficiently compete for the binding of ribosomal components to both 6AP and GA beads (at least as efficiently as 6AP and GA themselves), CPZ was not, as observed for 6APi and GAi, derivatives of 6AP and GA that are inactive as antiprion drugs. These results suggest that QC, but not CPZ, could share at least some binding sites with 6AP and GA on the large rRNA. The fact that CPZ was unable to compete for the binding of rRNA on 6AP and GA beads suggests that either it has other biological target(s) or it also targets the ribosome and possibly also the large rRNA but on different and nonoverlapping binding sites than 6AP and GA.

CONCLUSION

In the present study, we have used a simple chemical spacer arm easy to introduce by Mitsunobu reaction. Therefore, the introduction of the linker requires the synthesis of phenols obtained from their benzyl ether precursors compatible themselves with the various reaction conditions used in the synthesis of the tricyclic phenanthridine moitie. This spacer arm can be considered less hydrophilic than classical polyoxyethylenic linkers, thus greatly facilitating the purification of the conjugates by column chromatography. The two hydrophobic parts of the spacer should not be able to establish interactions either with the bioactive molecule or with the hydrophilic Sepharose beads. The amide bond in the middle of the linker reduces the flexibility of the spacer. To the best of our knowledge, the deprotection of functional groups after grafting on the beads has not been previously reported. Using this procedure, we synthesized affinity chromatography matrices for both 6AP and GA, two antiprion drugs active from yeast to mammals. The identification of the protein folding activity of the ribosome as a common target of both compounds suggests the intriguing possibility that this largely unknown function borne by the large rRNA could be involved in prion propagation. Finally, it has been recently found that PrP^C is an amyloid-β oligomers receptor (*38*).

Because amyloids β-fibers are found in the brains of people suffering from Alzheimer's disease, this finding enhances the interest of studies aiming at deciphering the cellular mechanisms involved in the appearance and/or propagation of prion-based diseases.

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