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Solid-Phase Organic Tagging Resins for Labeling Biomolecules by 1,3-Dipolar Cycloaddition: Application to the Synthesis of a Fluorescent Non-Peptidic Vasopressin Receptor Ligand

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Abstract: Two novel solid-phase organic tagging (SPOrT) resins were synthesized to facilitate the labeling of peptides and small organic compounds with a fluorescent probe. Both resins were obtained from the commercially available backbone amide linker (BAL) resin. Following the solid-phase synthesis of model compounds, a tripeptide and benzazepine, the fluorescent probe derived from Lissamine Rhodamine B was incorporated through Cu^{I}-catalyzed 1,3-dipolar cycloaddition. Final cleavage in acidic media enabled access to both types of molecules in good yield with high purity. The SPOrT resin was successfully applied to the preparation of the first non-peptidic fluorescent compound with a nanomolar affinity for the human vasopressin V_2 receptor (V_2R) subtype. This molecule will find application in binding assays that use polarization or fluorescence resonance energy-transfer (FRET) techniques. The SPOrT resins are also well suited for other tags and the parallel synthesis of a fluorescently tagged library for protein screening.

Keywords: click chemistry · cycloaddition · fluorescent probes · receptors · solid-phase synthesis

Introduction

Fluorescent techniques have proven to be extremely powerful tools to probe the structure and function of proteins. Numerous applications exist that range from biophysical characterization to fluorescent imaging of membrane proteins in living cells.[1] Fluorescence resonance energy-transfer (FRET) between a fluorescent donor–acceptor pair represents a convenient method to investigate intra- and intermolecular association processes both in vitro and in vivo.[2] The fluorescence polarization technique is also a very useful and convenient tool for binding studies and can advantageously be used for high-throughput screening of a fluorescently tagged library.[3] More generally, fluorescent-based techniques are especially appealing because of the potential sensitivity and specificity that can be achieved. In addition, fluorescent probes represent a safe and versatile alternative to radioligands, thus eliminating issues related to the handling and disposal of radioactive materials.

Nevertheless, the prerequisite for the application of such techniques is to design and synthesize fluorescent peptides or small-molecule-based ligands that retain the pharmacological profile of the nonlabeled probes. Several methods are currently available to label biomolecules. However, they generally suffer from limitations, such as the need for protecting groups and the separation of the product from excess reagent and by-products, which hamper the synthesis of fluorescent probes in high yield and purity. Therefore, there is still a definite need for a generic and highly flexible strategy to access to fluorescent molecules.

Herein, we describe a convenient and straightforward solid-phase approach to prepare both fluorescent peptides

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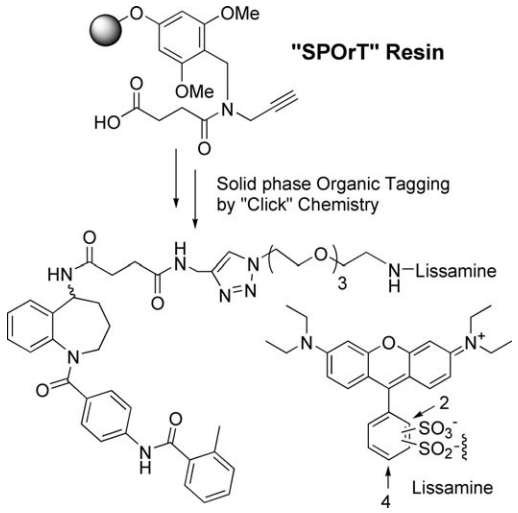
and fluorescent small organic compounds by using so-called solid-phase organic tagging (SPOrT) resins. This method combines the advantages of solid-phase chemistry with those of the Cu^{I}-catalyzed 1,3-dipolar cycloaddition, referred to as "click" chemistry.[4] To highlight the efficacy of the two novel resins, fluorescent 2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine (1-benzazepine) and a tripeptide were synthesized as model compounds in high yield and purity. In our program aimed at the design and synthesis of novel antagonists of arginine–vasopressin (AVP) receptors by using FRET based-assay, the SPOrT resin was successfully applied to the preparation of the first fluorescent non-peptidic ligand of the human vasopressin V_2 receptor (V_2R) subtype (Scheme 1).



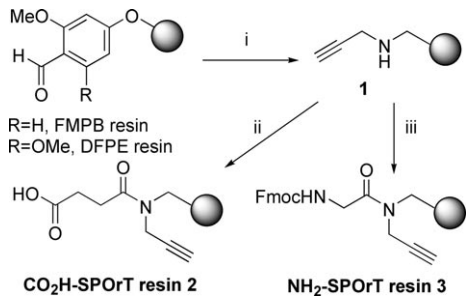
Scheme 1. Fluorescent 2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine derivatives synthesized using the SPOrT resin.

Results and Discussion

Based on the recent results obtained with the muscarinic M1 receptor,[2b] we devised a strategy that enabled the rapid solid-phase assembly of a fluorescent probe derived from Lissamine Rhodamine B, linked with either peptides or small molecules through a polyethylene glycol (PEG) spacer. This latter species should be long enough to limit the perturbation of ligand affinity. In addition, it should increase the solubility of fluorescent molecules in aqueous media. Our strategy entailed three parts: 1) the preparation of the SPOrT resins consisted of the solid-phase incorporation of an alkyne moiety on a backbone amide linker (BAL) resin, followed by the introduction of either an amine or carboxylic acid group to provide CO_2H– and NH_2–SPOrT resins **2** and **3** (Scheme 2); 2) the subsequent solid-phase elongation of peptides or small organic compounds; 3) conjugation of the fluorescent probe performed through a solid-phase Cu^{I}-catalyzed 1,3-dipolar cycloaddition by using a PEG—azido spacer bearing Lissamine Rhodamine B, a fluorescence dye with a high molar extinction coefficient (Scheme 3 and Scheme 4). The alkyne group is supposed to be stable through the solid-phase synthesis and enables the incorporation of the probe at the very last stage.

The preparation of CO_2H– and NH_2–SPOrT resins 2 and 3: We decided to immobilize the alkyne moiety on a solid support[5] to avoid the potential risk of the cross-coupling of alkynes in solution, such as the Glaser or Strauss coupling. The commercially available 4-(4-formyl-3-methoxyphenoxy)-butyryl (FMPB) resin was treated with propargylamine according to the protocol developed by Barany and co-workers, namely, using $NaBH_3CN$ in DMF/AcOH (99:1) at room temperature.[6] However, following these conditions, the reaction was not complete, as shown by the positive result to a test with 2,4-dinitrophenylhydrazide (DNPH).[7] To accelerate the kinetics of the reductive amination, the solubility of the reagents was improved by adding MeOH (19%) to the resin, and the resulting mixture was heated to 60°C. After 16 h, the negative result to a test with chloranil was indicative of a complete, successful reaction.[8]

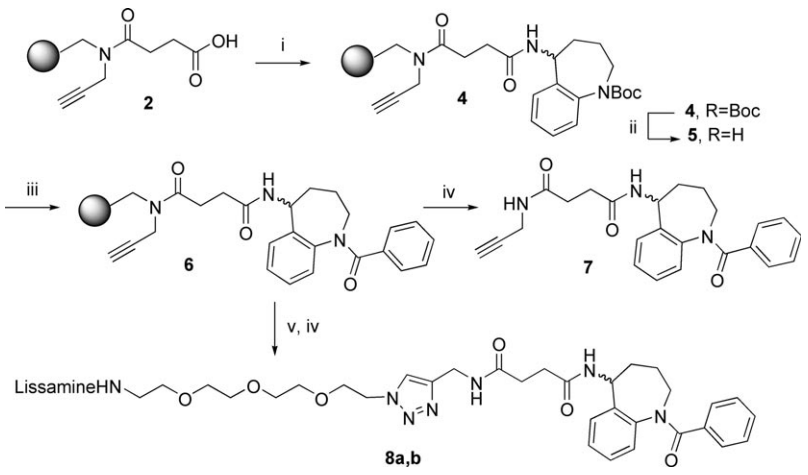
To access resins **2** and **3**, supported propargylamine **1** was treated in presence of either succinimic anhydride or Fmoc–Gly–OH in presence of a coupling agent. Opening of the anhydride was efficiently performed in the presence of pyridine and 4-DMAP to provide resin **2**. The presence of the carboxylic acid group was confirmed by using the malachite green test (Scheme 2).[9] The *N*-Fmoc-protected glycine moiety was successfully introduced by activation with PyBOP in situ in CH_2Cl_2/DMF (1:1) to give resin **3**.



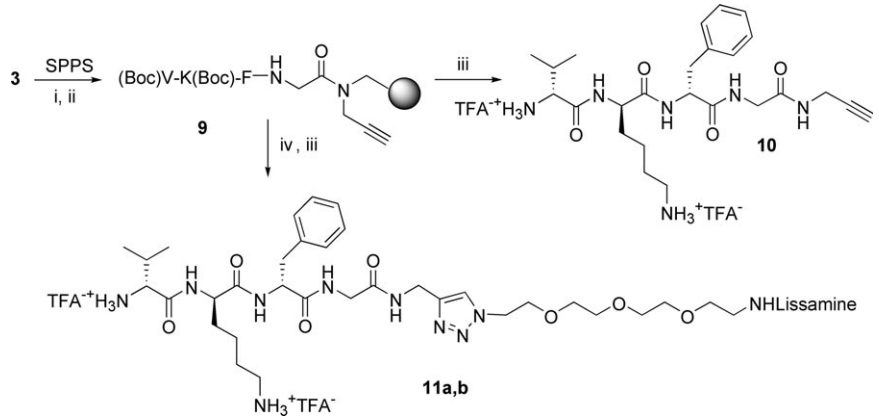
Scheme 2. Preparation of the SPOrT resins **2** and **3**. i) Propargylamine (10 equiv), $NaBH_3CN$ (10 equiv), DMF/MeOH/AcOH (80:19:1), 60°C, overnight; ii) succinic anhydride (25 equiv), 4-DMAP (1 equiv), pyridine (4 equiv), DMF, RT, overnight; iii) Fmoc-Gly-OH (4 equiv), PyBOP (4 equiv), DIEA (12 equiv), NMP, RT, overnight. 4-DMAP = 4-dimethylaminopyridine, Fmoc = 9-fluorenylmethoxycarbonyl, NMP = 1-methyl-2-pyrrolidone, PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

Solid-phase synthesis of model biomolecules: With resins **2** and **3** in hand, their potential to provide rapid access to fluorescent biomolecules (small organic compounds and peptides) was investigated by considering two types of model compounds: a fluorescent 1-benzazepine scaffold extensively studied by us[10] and a tripeptide VFK, which contained two basic sites susceptible to hampering the final cleavage from the BAL resin.

Resin-bound 1-benzazepine **6** was prepared in two steps from CO_2H–SPOrT resin **2** (Scheme 3). The 2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepin-5-amine scaffold was obtained in a six-step process starting from the commercially available methyl-2-aminobenzoate.[10] This scaffold was then anchored to resin **2** through an amide linkage by activation with PyBOP in situ. After 16 h at room temperature, a negative result to a test with malachite green was found. The endocyclic nitrogen atom of the resin-bound benzazepine **4** was deprotected in the presence of TMSOTf and triethylamine (resin **5**) and subsequently acylated with benzoyl chloride to provide resin **6** (Scheme 3). Starting from NH_2–SPOrT resin **3**, the resin-bound model compound tripeptide VKF–OH **9** was synthesized by following a classical Fmoc/*tert*-butyl strategy and activation by HBTU (Scheme 4).



Scheme 3. Solid-phase synthesis of the model compound benzazepine using CO_2H–SPOrT resin **2**. i) Aminobenzazepine (3 equiv), PyBOP (3 equiv), DIEA (4 equiv), DMF; ii) TMSOTf (3 equiv), Et_3N (1.5 equiv), CH_2Cl_2, RT, 15 min; iii) benzoyl chloride (5 equiv), pyridine (10 equiv), 4-DMAP (0.05 equiv), CH_2Cl_2, RT, overnight; iv) TFA/$H_2$0 (95:5), RT, 3 h; v) **11** (3 equiv), CuI (5 equiv), piperidine/DMF (1:5), 30 °C, 6 h. Boc = *tert*-butyloxycarbonyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyltriflate.



Scheme 4. Solid-phase synthesis of the model tripeptide compound using NH_2–SPOrT resin **3**. i) Piperidine/DMF (1:5), RT, 2×20 min; ii) amino acid (4 equiv), HBTU (4 equiv), DIEA (12 equiv), NMP, RT, 2 h; iii) TFA/$H_2$0 (95:5), RT, 3 h; iv) **11** (3 equiv), CuI (5 equiv), piperidine/DMF (1:5), 30 °C, 6 h. HBTU = 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

To evaluate the efficacy of the solid-phase reactions, samples of resin **6** and **9** were treated in TFA/H_2O (95:5) for 3 h to provide alkyne–benzazepine **7** and tripeptide **10** in 61 and 38 % yield, respectively (91 % purity for both, as estimated by LC-MS at $\lambda = 224$ nm). In an attempt to increase the yield of the alkynes, the importance of the time of cleavage and the nature of the BAL resin linker were investigated (Table 1). The more acid-sensitive 2-(3,5-dimethoxy-4-formylphenoxy)-ethyl polystyrene (DFPE) resin gave better yields and higher purities than the FMPB resin. Whereas the cleavage step was complete after 16 hours in TFA/water (95:5) with the FMPB resin (Table 1, entry 2), only 3 h was necessary for the DFPE resin. Alkyne–benzazepine **7** and alkyne–tripeptide **10** were obtained in 95 and 57 % yield (96 and 94 % purity), respectively (Table 1, entry 3).

Table 1. Influence of the nature of the BAL resin and the time of cleavage on alkynes preparation.

Entry	Resin(s)	Cleavage[a] time [h]	Yield[b] [%]/Purity[c] [%] Alkynes **7**	**10**	Lissamine derivatives[d] **8a,b**	**11a,b**
1	FMPB	3	61/91	38/91	23/82	10/77
2	FMPB	16	89/86	50/98	51/85	26/82
3	DFPE	3	95/96	57/94	84/89	50/91
4	DFPE	16	90/95	56/92	78/88	49/90

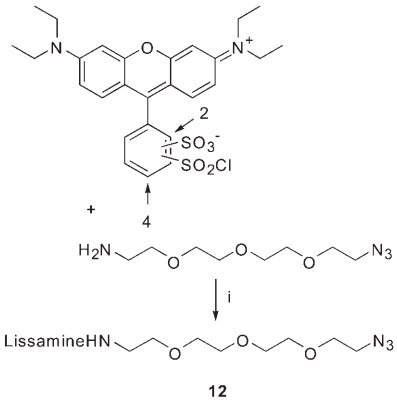
[a] Performed in TFA/water (95:5). [b] Determined by weight of the crude products based on the initial BAL resin loading. [c] Determined by RP-HPLC analysis of the crude product at $\lambda = 254$ nm. [d] Obtained as a mixture of *ortho* and *para* isomers.

It is of note that Lissamine Rhodamine B was found to be highly stable under the conditions for acidic cleavage, thus showing the suitability of this probe for the solid-phase synthesis of fluorescent compounds. Alkyne–peptide **10** was obtained in a lower yield than benzazepine **7**. This result can be ascribed to the loading of the Fmoc–Gly–DFPE resin **3**. Indeed, Fmoc quantification[11] gave a loading of 0.77 versus 0.92 mmol g^{-1} for the commercially available DFPE resin. Despite monitoring by colorimetric tests, the reductive amination/acylation reactions were not complete (83 % yield

based on the loading of the DFPE resin). With resin-bound alkynes **6** and **9** in hand, we investigated the solid-phase incorporation of the fluorescent probe through click chemistry.

Solid-phase click chemistry: The generation of [1,2,3]triazoles by the 1,3-dipolar cycloaddition of alkynes to azides proceeds at room temperature in the presence of Cu^I as a catalyst. This transformation has been successfully used for the fluorescent solution-phase labeling of virus capsid proteins,[12] the cell surface of *Escherichia coli*,[13] and activity-based protein profiling.[14] We used it for the labeling of the muscarinic M1 antagonist pirenzepine for the FRET-based binding assay.[15] It has been shown that the 1,4-disubstituted 1,2,3-triazoles can serve as transoid amide-bond mimics in natural compounds without compromising biological activity.[16] The solid-phase version of click chemistry was recently described for the synthesis of peptidomimetics,[17] multiple-labeled carbohydrate oligonucleotides,[18] and functionalized resins.[19] However, the incorporation of fluorescent probes on resins by click chemistry has been much less studied. To the best of our knowledge, only one example to date has related the surface functionalization of resins (Wang and Merrifield) with dyes using a Huisgen [2+3] cycloaddition reaction, performed at 70°C under nitrogen.[20]

Our approach relies on the solid-phase incorporation of a Lissamine Rhodamine B derivative by employing the catalytic version of the Huisgen [2+3] cycloaddition. To achieve our goal, Lissamine-$(PEG)_3$-azido **12** was synthesized by treating Lissamine Rhodamine B sulfonyl chloride, commercially available as a mixture of *ortho* and *para* isomers, with 11-azido-3,6,9-trioxaundecan-1-amine in CH_2Cl_2/DMF (4:1) in the presence of triethylamine and a catalytic amount of 4-DMAP (Scheme 5). The PEG chain was selected to increase the solubility of the Lissamine, and thus favor both the reaction and the elimination of excess Lissamine from the solid support. Compound **12** was isolated in 69% yield as a 1:3 mixture of *ortho* and *para* isomers, as determined by reverse-phase-HPLC (RP-HPLC; column: C18, eluent: water,



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Scheme 5. Preparation of lissamine-$(PEG)_3$-azido **12**. i) 11-Azido-3,6,9-trioxaundecan-1-amine (1 equiv), Et_3N (3 equiv), 4-DMAP (0.1 equiv), CH_2Cl_2/DMF (1:1), RT, overnight (69%).

detection: $\lambda = 254$ nm) and ^1H NMR spectroscopic analysis.[21]

Starting from recently described solid-phase click chemistry conditions,[16] azido **12** was treated with both alkyne-bound resins **5** and **9** in the presence of an excess of CuI in DMF/piperidine (4:1, v/v). Optimization of the reaction demonstrated that the best conversion was obtained with three equivalents of **12** after 6 h at 30°C. It is of note that the addition of ascorbic acid or tris(carboxyethyl)phosphine, generally used to stabilize the Cu^I species, did not permit an increase in the yield of the reaction. The influence of the time of cleavage and the nature of the BAL linker (FMDP versus DFPE) on both the yield and purity was also investigated (Table 1).

As previously shown for alkynes **7** and **10**, the best results were obtained by treating the fluorescent compound bound DFPE resin in TFA/H_2O (95:5) for 3 h (Table 1, entry 3). Under these conditions, the fluorescent benzazepine **8** was obtained in 84% yield (89% purity) as a mixture of *ortho* and *para* isomers **8a**,**b** in a 1:3 ratio. Following a six-step solid-phase process, the fluorescent tripeptide **11** was obtained in 50% yield (91% purity) as a mixture of *ortho* and *para* isomers **11a**,**b**. The RP-HPLC profile of the crude mixture highlights the efficacy of the process (Figure 1). The alkynes and Lissamine-$(PEG)_3$-azido **12** were not detected by LC-MS analysis of the crude product, thus showing both the completion of the solid-phase 1,3-cycloaddition reaction and the efficient removal of the starting material azido **12** from the resin prior to the cleavage. Owing to the efficacy of the solid-phase click process and the excellent purity of the crude mixture, fluorescent compounds prepared according to this method could be used directly for biological evaluation without any additional purification.

Application of the CO_2H–SPOrT resin to the synthesis of a novel fluorescent V_2R ligand: FRET-based assays have been

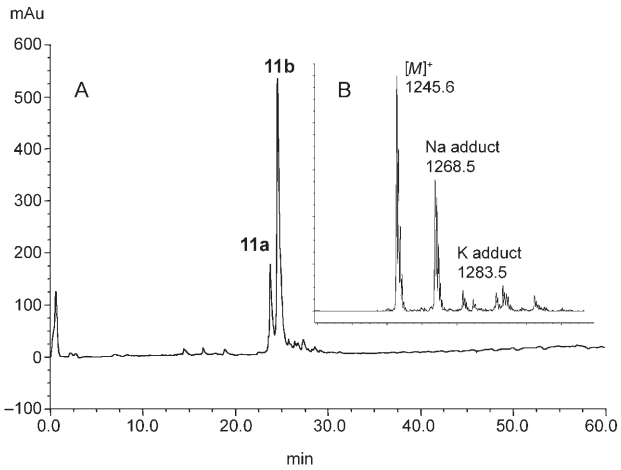
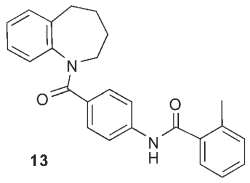
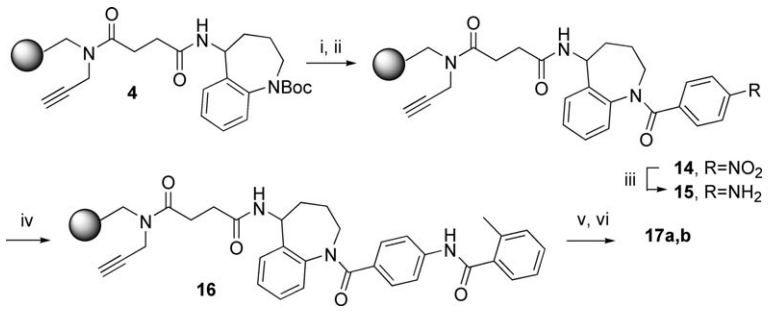


Figure 1. RP-HPLC chromatogram and positive ESI-MS spectrum of the crude model compound tripeptide **11a**,**b**. Chromatographic conditions: C18 symmetry shield column (4.6×150 mm). Flow rate: 1 mL min^{-1}, buffer A: 0.1% aqueous TFA, buffer B: 0.1% TFA in CH_3CN, gradient: 0–100% B over 60 min, detection: $\lambda = 220$ nm.

extensively used by us to study G protein-coupled receptors (GPCRs).[2a–c] In a program aimed at accelerating the discovery of original ligands of vasopressin and oxytocin receptors, we were interested in preparing a fluorescent probe with good binding affinity for V_2R.



Starting from 3-methyl-4′-[(2,3,4,5-tetrahydro-1*H*-1-yl)carbonyl]benzanilide (**13**), which was reported to bind to the V_2 receptor with a nanomolar affinity (K_i = 7.2 nM on rabbit kidney membranes),[22] the solid-phase preparation of the corresponding fluorescent compound was investigated with CO_2H–SPOrT resin **2** (Scheme 6). After the incorporation of the aminobenzazepine scaffold on the DFPE solid support (i.e., resin **4**), the endocyclic secondary amine group was deprotected and acylated with *para*-nitrobenzoyl chloride in the presence of pyridine and 4-DMAP. After the reaction mixture was stirred overnight, a negative result to the colorimetric test with Chloranil was indicative of a quantitative reaction (i.e., resin **14**). Reduction of the nitro group was then investigated by using tin chloride in DMF at 60 °C.[23]



Scheme 6. Solid-phase synthesis of fluorescent benzazepine **17a**,**b** using the CO_2H–SPOrT resin. i) TMSOTf (3 equiv), Et_3N (1.5 equiv), CH_2Cl_2, RT, 15 min; ii) 4-nitrobenzoyl chloride (5 equiv), pyridine (10 equiv), 4-DMAP (0.1 equiv), CH_2Cl_2, RT, overnight; iii) $SnCl_2{\cdot}2H_2O$, DMF, RT, overnight; iv) *ortho*-toluyl chloride (5 equiv), DIEA (10 equiv), 4-DMAP (1 equiv), CH_2Cl_2, RT, overnight; v) click chemistry; vi) TFA/H_20 (95:5), RT, 3 h.

Under these conditions, the expected resin-bound amine **15** was obtained together with an unidentified by-product. Further optimization showed that the side reaction could be avoided by performing the reduction reaction at room temperature. *N*-Acylation of the weakly nucleophilic amine with the *ortho*-toluyl acyl moiety was also found to be a critical step in the synthesis. Various coupling agents, such as PyBOP, HATU, PyBrOP, and tetramethylfluoroformamidinium hexafluorophosphate (TFFH) in presence of *ortho*-toluyl acid failed to provide a complete reaction. Ultimately, the use of the corresponding acyl chloride with 4-DMAP, the Hünig base, in CH_2Cl_2 was found to provide the best conditions under which to access the benzazepine-bound resin **17**.

Click chemistry in presence of Lissamine-$(PEG)_3$-azido derivative **12** followed by treatment of the resin with TFA gave access to Lissamine benzazepine **17** as a mixture of *ortho* and *para* isomers **17a**,**b** (1:1.6, as shown by LC-MS analysis). RP-HPLC purification on a C18 column enabled isolation of both isomers in 16 and 28% yield for the *ortho* and *para* isomers, respectively (nine steps). The retention times of both isomers indicate that the *para* isomer is more lipophilic than the *ortho* isomer (Table 2). The absorption spectra and molar extinction coefficients ε were recorded in a 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer containing a final concentration of 0.5% dimethyl sulfoxide (DMSO; Table 2). Both isomers have similar maximum absorptions (λ = 572 and 574 nm for the *ortho* and *para* isomers, respectively) and very close molar extinction coefficients (ε = 65000 and 69000 $M^{-1}\,cm^{-1}$ for the *ortho* and *para* isomers, respectively).

Affinities for the V_2 receptor: The affinity of compound **13** and isomers **17a**,**b** for the human V_2 receptor were determined by competition experiments against [^{3}H]-AVP (AVP = arginine vasopressin), as described previously[24] (Table 2). The affinity of **13** for the human receptor was found to be close to that determined for rabbit kidney membranes (K_i = 2.68 versus 7.2 nM). The *ortho* isomer **17a** exhibits a K_i value that is threefold higher than the *para* isomer **17b** (187 versus 54 nM). The presence of the PEG spacer and the Lissamine moiety disturbs binding to the V_2 receptor. In addition, the position of the link between the fluorophore and the benzazepine core (*ortho* versus *para* isomers) affects the

Table 2. Characterization and properties of fluorescent compounds **17a**,**b**.

Entry	Compound	t_R[b] [min]	λ_{max} [nm]	$\varepsilon_{\lambda max}$ [$10^3\,M^{-1}\,cm^{-1}$]	K_i [nM][c]
1	[^{3}H]AVP	–	–	–	1.36 ± 0.45
2	**13**	–	–	–	2.68 ± 0.38
3	**17a**[a]	3.91	572	65	187.7 ± 24.7
4	**17b**[a]	4.03	574	69	54.3 ± 6.6

[a] Absorption parameters measured in a 50 nM HEPES, pH 7.5 buffer containing a final concentration of 0.5% DMSO (v/v). [b] Retention times were obtained on a Chromolith SpeedROD column (50 × 4.6 mm, C18) under the experimental conditions described in the Experimental Section. [c] The inhibition constants K_i of fluorescent compounds of the human vasopressin V_2 were determined on CHO cell membranes by competition binding assays (displacement of radioactive [^{3}H]AVP); the results are expressed as mean ± SEM of three separate experiments performed in triplicate (SEM = standard error of the mean).

affinity of the ligand for the receptor. Nevertheless, the ligand derivatized with Lissamine at the *para* position still displays an affinity that is compatible with fluorescence-based assays by using either polarization or FRET techniques.[25] It is of note that this molecule represents the first fluorescent non-peptidic ligand for V_2R described to date.

Conclusion

Fluorescent peptides or small-molecule-based ligands have proven to be extremely powerful tools with which to investigate receptor function and signal-transduction processes, as well as for applications in the field of screening for novel therapeutic compounds. Herein, we have described two novel SPOrT resins to facilitate the synthesis of such molecules. The method is rapid and straightforward and enables the chemoselective and quantitative labeling of both peptides and small organic compounds by using click chemistry. The final products were obtained in good yield with high purity. This strategy was successfully applied to the preparation of the first non-peptidic fluorescent compound with nanomolar affinity for the human vasopressin V_2 receptor. This molecule will find application in structural studies of receptors and the discovery of original ligands by using fluorescence polarization techniques. In addition, SPOrT resins are particularly appealing for the parallel synthesis of fluorescent chemical libraries. Such a program is in progress to identify GPCR orphan ligands using FRET-based assays, and the results will be reported in due course.

Experimental Section

General: 4-(4-Formyl-3-methoxyphenoxy)butyryl aminomethylated polystyrene resin (FMPB AM; 100—200 mesh, 0.74 mmol g^{-1}) and 2-(3,5-dimethoxy-4-formylphenoxy)ethyl polystyrene resin (DFPE; 100—200 mesh, 0.92 mmol g^{-1}; BAL-type resin) were purchased from Novabiochem; 11-azido-3,6,9-trioxaundecan-1-amine was obtained from Fluka; (±)-5-amino-1-*tert*-butoxycarbonyl-2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepine (1-benzazepine) was obtained as previously described.[10] Solid-phase reactions conducted at room temperature were performed in polypropylene tubes equipped with polyethylene frits and polypropylene caps using an orbital-agitator shaking device. Solid-phase reactions at 60 °C were conducted in sealed glassware tubes using the Chemflex rotating oven from Robbins Scientific as the shaking device.

^{1}H NMR spectra were recorded at 200, 300, and 500 MHz on a Bruker Advance spectrometer. Chemical shifts are reported in parts per million (ppm), and the coupling constants *J* are reported in hertz (Hz). LC-MS spectra were obtained on a ZQ (Z quadripole) Waters/Micromass spectrometer equipped with an X-Terra C18 column (4.6×50 mm, 3.5 μm) using the electrospray ionization mode (ESI). HRMS spectra were obtained on a MicroTof mass spectrometer from Bruker using the ESI mode and a time-of-flight analyzer (TOF). Thin-layer chromatography was performed on silica gel 60 F_{254} plates from Merck. Flash chromatography was performed on silica gel 60 (230–400 Mesh ASTM) from Merck. Analytical HPLC analyses were performed on a Chromolith SpeedROD column (50×4.6 mm, C18) from Merck under the following conditions: flow rate: 7 mL min^{-1}; buffer A: 0.1% aqueous TFA, buffer B: 0.1% TFA in CH_3CN; gradient: 0% buffer B for 1 min then 0–100% buffer B over 5 min; detection: λ=220/254 nm. The retention times t_R from analytical RP-HPLC are reported in minutes.

***N*-[10-[4 (and 2)-{[(2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethyl)amino]-sulfonyl}-2 (and 4)-(methylsulfonyl)phenyl]-7-(diethylamino)anthracen-2-(9*H*)-ylidene]-*N*-ethylethanaminium (12)**: 11-Azido-3,6,9-trioxaundecan-1-amine (200 μL, 1 mmol) was dissolved in CH_2Cl_2/DMF (4:1) and Et_3N (421 μL, 3 mmol) in the presence of 4-DMAP (12.2 mg, 0.1 mmol) in an argon atmosphere. The resulting mixture was stirred at 0 °C for 15 min. Lissamine Rhodamine B sulfonyl chloride (577 mg, 1 mmol) was added in portions over 20 min. The solution was allowed to warm up to room temperature and stirred overnight. Crude reaction mixture was concentrated under reduced pressure. Compound **12** was isolated by flash chromatography with a step gradient from 5 to 10% MeOH in CH_2Cl_2 and obtained as a red-dark solid (526 mg, 69%, *ortho* and *para* isomers, 1:1.8 ratio, respectively). R_f=0.32 (*para* isomer) and 0.28 (*ortho* isomer) (CH_2Cl_2/MeOH 9:1); ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 300 MHz): δ=8.56 (d, *J*=1.8 Hz, 0.63H), 8.43 (d, *J*=1.8 Hz, 0.37H), 8.10 (dd, *J*=7.8, 1.8 Hz, 0.37H), 7.88 (dd, *J*=7.8, 1.8 Hz, 0.63H), 7.15 (dd, *J*=7.8, 2.8 Hz, 0.37H), 7.01 (dd, *J*=9.5, 3.4 Hz, 0.37H), 7.02–6.98 (m, 2H), 6.79–6.68 (m, 2H), 6.63–6.59 (m, 2H), 3.55–3.19 (m, 20H), 3.08 (t, *J*=5.3 Hz, 1.26H), 2.91 (t, *J*=5.3 Hz, 0.74H) 1.21–1.13 ppm (m, 12H); ^{13}C NMR ($CDCl_3$/CD_3OD 9:1, 75 MHz): δ=157.6, 157.5, 157.2, 155.4, 155.3, 148.4, 146.5, 142.0, 133.4, 132.6, 132.0, 131.1, 130.5, 130.0, 129.5, 127.3, 126.6, 125.7 ppm; RP-HPLC purity: >95%; HRMS: calcd for $C_{35}H_{47}N_6O_9S_2$ 759.2840; found: 759.2850.

Standard washing protocol: Resin washings were performed with DMF (3×1 min), MeOH (3×1 min), and CH_2Cl_2 (3×1 min).

Synthesis of resin-bound alkyne 1: Propargylamine (158 μL, 2.3 mmol) in DMF/MeOH/AcOH (2.5 mL; 80:19:1, v/v/v) and $NaBH_3CN$ (144.5 mg, 2.3 mmol) were added to DFPE resin (210 mg, 0.92 g mol^{-1}, 0.23 mmol). The reaction mixture was heated at 60 °C overnight, allowed to cool to room temperature, and filtered. The resin was washed following the standard washing procedure and dried in vacuo. Complete loading of propargylamine onto the DFPE resin was verified by a negative result of a test with DNPH.[7]

Synthesis of CO_2H−SPOrT resin 2: A solution of succinic anhydride (575.4 mg, 57.5 mmol) in DMF (1.55 mL), pyridine (74 μL, 0.92 mmol), and 4-DMAP (28.1 mg, 0.23 mmol) were added to the resin-bound alkyne **1** (0.23 mmol) preswollen in DMF. The resulting mixture was shaken overnight at room temperature. The resin was filtered, washed, and dried in vacuo to give resin **2**. Complete reaction was verified by a negative result of a test with Chloranil, and the presence of CO_2H group by the positive result to a test with Malachite green.

Synthesis of Fmoc-NH-SPOrT resin 3: Fmoc-Gly-OH (219 mg, 0.73 mmol) dissolved in NMP (1:1, 1.74 mL), PyBOP (383 mg, 0.73 mmol), and the Hünig base (385 μL, 2.2 mmol) were added to the resin-bound alkyne **1** (0.184 mmol) preswollen in NMP (1:1, v/v). After the reaction mixture had been stirred overnight at room temperature, a negative result to a test with chloranil was indicative of the completion of the reaction. The resin was washed and dried in vacuo.

Procedure for the loading of the 1-benzazepine scaffold onto CO_2H−SPOrT resin 2: A solution of 1-benzazepine (144.8 mg, 0.552 mmol) in DMF (1 mL), PyBOP (287.2 mg, 0.552 mmol), and the Hünig base (128.2 μL, 0.736 mmol) were added to DFPE resin (0.184 mmol). After the reaction mixture was shaken overnight at room temperature, the resin was washed. A negative result for a test with Malachite showed the completion of the coupling. Resin **4** was dried in vacuo.

General procedure for selective 1-benzazepine Boc deprotection: A freshly prepared solution of TMSOTf (109 μL, 0.552 mmol) and Et_3N (38.8 μL, 0.276 mmol) in anhydrous CH_2Cl_2 (4 mL) were added to resin-bound *N*-Boc benzazepine **4** (0.184 mmol) preswollen in anhydrous CH_2Cl_2. The reaction mixture was shaken at room temperature for 15 min, filtered, washed (CH_2Cl_2 (3×), MeOH (3×), and CH_2Cl_2), and dried in vacuo to give resin **5**.

Synthesis of resin-bound benzazepine 6: A solution of benzoyl chloride (39.7 μL, 342.1 μmol) in CH_2Cl_2 (924 μL), 4-DMAP (0.5 M in CH_2Cl_2, 7 μL), and pyridine (55 μL, 684 μmol) were added to resin-bound benza-

zepine **5** (68.4 μmol) preswollen in CH_2Cl_2. The reaction mixture was shaken for one night at room temperature, washed, and dried in vacuo.

General procedure for final deprotection and cleavage steps: The resin was treated in the presence of TFA/H_2O (95:5 v/v) for 3 h at room temperature, filtered, washed with CH_2Cl_2 (2×) and MeOH (2×). The combined filtrates were mixed, and evaporated to dryness in vacuo. The crude residue was dissolved in acetonitrile/H_2O (1:1, v/v) and lyophilized.

Synthesis of *N*-(1-benzoyl-2,3,4,5-tetrahydro-1*H*-benzo[*b*]azepin-5-yl)-*N*′-prop-2-ynyl-succinamide (7): Resin **6** (22.0 μmol) was treated with TFA/H_2O (95:5 v/v). Compound **7** was isolated by RP-HPLC on a C18 symmetry shield column as a white solid (6.3 mg, 71%). t_R = 3.03 min; ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 7.35 (dd, J = 7.4, 1.8 Hz, 2H), 7.20–7.08 (m, 5H), 6.89 (td, J = 7.4, 1.8 Hz, 1H), 6.57 (d, J = 7.4 Hz, 1H), 5.33 (bdd, J = 11.0, 3.4 Hz, 1H), 4.64–4.46 (m, 1H), 3.98 (bt, J = 2.2 Hz, 2H), 3.0–2.96 (m, 1H), 2.70–2.44 (m, 5H), 2.18 (t, J = 2.6 Hz, 1H), 2.10–1.88 (m, 1H), 1.76–1.56 ppm (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 172.6, 172.2, 170.4, 140.7, 138.5, 135.7, 129.9, 128.9, 128.7, 128.4, 127.9, 127.7, 127.6, 124.1, 71.5, 66.0, 50.7, 49.6, 49.2, 48.7, 46.7, 31.7, 29.3 ppm; RP-HPLC purity: >95%; HRMS: calcd for $C_{24}H_{26}N_3O_3$ 404.1969; found: 404.1970.

Synthesis of resin-bound protected peptide 9: The model peptide compound was elaborated on the Fmoc-NH-SPOrT resin **3** (184 μmol) by using a classical Fmoc/*tert*-butyl strategy. Removal of the Fmoc group was performed with DMF/piperidine (4:1, v/v) for 20 min. Fmoc amino acids (Fmoc-L-Phe-OH, Fmoc-L-Lys(Boc)-OH, and Boc-L-Val-OH; 730 μmol) were coupled by the addition of HBTU (279 mg, 730 μmol), *N*,*N*-diisopropylethylamine (DIEA; 385 μL, 2.2 mmol), and NMP (1.74 mL) for 2 h at room temperature. Completion of the reaction was monitored by using trinitrobenzenesulfonic acid (TNBS) as a test.[26] The coupling of the amino acids and deprotection of the Fmoc group were followed by washing with DMF (3×) and CH_2Cl_2 (3×).

Synthesis of H-VKFG-NHCH$_2$-CH 10: Resin **9** (23.7 μmol) was treated with TFA/H_2O (95:5 v/v) for 3 h at room temperature. Following a freeze/drying step, peptide **10** was isolated by RP-HPLC on a C18 symmetry shield column as a white powder (6.4 mg, 38%). t_R = 2.43 min; HRMS: calcd for $C_{25}H_{39}N_6O_4$ 487.3027; found: 487.3041; RP-HPLC purity: >95%.

General procedure for the solid-phase click chemistry: Resin-bound alkyne (46 μmol) was preswollen in DMF/piperidine (4:1, v/v). In two separate vials, CuI (44.3 mg, 230 mmol) and azido **12** (105.8 mg, 139 μmol) were dissolved in DMF/piperidine (825 μL; 4:1, v/v) using an ultrasonic bath. Both solutions were mixed altogether and placed for a further 5 min in an ultrasonic bath. The resulting mixture (1.65 mL) was added to the preswollen resin-bound alkyne. After the reaction mixture had been shaken for 6 h at 30°C, the resin was washed successively with DMF/piperidine (4:1, v/v; 3×), DMF (3×), CH_2Cl_2 (3×), CH_2Cl_2/MeOH (3×), MeOH (3×), and CH_2Cl_2 (3×) and dried in vacuo.

Synthesis of fluorescent 1-benzazepine 8a,b: Resin **6** (12.1 μmol) was submitted to click chemistry following the procedure described above. Following deprotection and cleavage in TFA/$H_2$0 (95:5), triazoles **8a** (*ortho* isomer) and **8b** (*para* isomer) were isolated by RP-HPLC on a C18 symmetry shield column as a red solid. **8a** (1.9 mg, 13%): t_R = 1.90 min; ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 500 MHz): δ = 8.57 (bs, 1H), 8.17 (d, J = 8.1 Hz, 1H), 7.78 (bs, 1H), 7.32–7.04 (m, 10H), 6.82 (bt, J = 10.0 Hz, 3H), 6.67 (bs, 2H), 6.49 (d, J = 7.4 Hz, 1H), 5.25 (bdd, J = 11.0, 3.4 Hz, 1H), 4.50 (bs, 1H), 4.41 (bs, 2H), 4.37 (bt, J = 5.0 Hz, 2H), 3.96–3.72 (m, 8H), 3.60–3.44 (m, 8H), 3.41 (t, J = 5.5 Hz, 2H), 3.27 (bs, 2H), 3.0 (t, J = 5.5 Hz, 1H), 2.63 (bt, J = 7.0 Hz, 2H), 2.55 (bt, J = 7.0 Hz, 2H), 2.08–1.96 (bs, 2H), 1.68–56 (bs, 2H), 1.25 ppm (m, 12H); ^{13}C NMR ($CDCl_3$/CD_3OD 9:1, 75 MHz): δ = 172.9, 172.3, 170.4, 157.6, 156.1, 155.5, 148.4, 140.9, 138.5, 135.4, 132.0, 131.4, 131.3, 130.7, 129.6, 129.5, 128.2, 128.0, 127.6, 127.5, 127.2, 125.7, 124.1, 113.9, 95.8, 70.2, 70.0, 69.9, 69.4, 69.0, 50.2, 50.0, 46.5, 45.8, 42.8, 34.6, 31.3, 25.1, 12.2 ppm; RP-HPLC purity: >95%; **8b** (4.8 mg, 33%): t_R = 2.017; ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 500 MHz): δ = 8.60 (bs, 1H), 7.92 (dd, J = 8.1, 1.1 Hz, 1H), 7.73 (bs, 1H), 7.22 (bd, J = 7.5 Hz, 2H), 7.17 (d, J = 7.5 Hz, 2H), 7.16–6.96 (m, 6H), 6.77 (t, J = 7.5 Hz, 1H), 6.71 (td, J = 7.5, 2.0 Hz, 2H), 6.61 (s, 2H), 6.44 (d, 7.50 Hz, 1H), 5.16 (dd, J = 11.0, 3.4 Hz, 1H), 4.43 (bs, 1H), 4.35 (bt, J = 4.5 Hz, 4H), 3.86 (bs, 8H), 3.74 (bt, J = 3.5 Hz, 2H), 3.62- 3.42 (m, 8H), 3.21 (bs, 2H), 3.11 (t, J = 5.5 Hz, 2H), 2.92 (bt, J = 11.5 Hz, 1H), 2.53 (bd, J = 6.0 Hz, 2H), 2.46 (bd, J = 6.0 Hz, 2H), 1.96–1.84 (m, 2H), 1.60–1.44 (m, 2H) 1.17 ppm (m, 12H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 172.8, 172.1, 170.3, 157.6, 156.8, 155.3, 146.0, 142.0, 140.0, 138.4, 135.2, 133.5, 132.5, 130.1, 129.5, 128.4, 128.0, 127.9, 127.5, 127.3, 127.1, 126.4, 123.9, 123.8, 123.7, 113.8, 113.3, 95.4, 70.1, 69.9, 69.4, 68.9, 50.2, 50.1, 50.0, 46.4, 45.6, 42.7, 34.4, 31.1, 31.0, 30.9, 27.9, 24.9, 12.3 ppm; RP-HPLC purity: >95%; HRMS: calcd for $C_{59}H_{72}N_9O_{12}S_2$ 1162.4736; found: 1162.4718.

Synthesis of fluorescent peptide 11a,b: Resin **9** (12.1 μmol) was submitted to the click chemistry procedure. Following deprotection and cleavage in TFA/$H_2$0 (95:5), triazoles **11a** (*ortho* isomer) and **11b** (*para* isomer) were isolated by RP-HPLC on a C18 symmetry shield column as a red solid. **11a** (1.7 mg, 10%): t_R = 3.39 min; RP-HPLC purity: >95%. **11b** (5.0 mg, 28%): RP-HPLC purity: >95%; HRMS: calcd for $C_{60}H_{85}N_{12}O_{132}S_2$ 1245.5795; found: 1245.5817.

Synthesis of fluorescent V$_2$R ligand 17a,b: 4-Nitrobenzoyl chloride (85 mg, 460 μmol) in CH_2Cl_2 (1.25 mL), pyridine (74 μL, 920 μmol), and 4-DMAP (0.5 M in CH_2Cl_2, 9.2 μL) were added to resin **5** (92 μmol). The reaction mixture was shaken for one night at room temperature. The completion of the reaction was verified by a negative result to a test with chloranil. Following the washing procedure, resin **14** was preswollen in DMF. A solution of $SnCl_2{\cdot}2H_2O$ (623 mg, 2.76 mmol) in DMF (1.5 mL) was added to the resin. Following shaking for one night at room temperature, the resin was washed with DMF/AcOH (3:1, v/v; 3×), DMF/Et_3N (9:1, v/v; 3×), DMF (3×), CH_2Cl_2 (3×), and MeOH (3×) and dried with Et_2O. Resin **15** was preswollen in CH_2Cl_2. *O*-Toluyl chloride (60 μL) in CH_2Cl_2 (1.35 mL) was added to the resin in presence of the Hünig base (160 μL, 920 μmol) and DMAP (11.2 mg, 92 μmol). The reaction mixture was shaken for one night at room temperature, after which the resin was filtered and washed. To ensure the completion of the reaction, the acylation step was repeated over 6 h, the resin was washed and dried in vacuo. The dried resin **16** (46 μmol) was submitted to the click chemistry protocol with azido **12** (106 mg, 139 μmol). A subsequent deprotection and cleavage in TFA/H_2O (95:5) gave compound **17a,b.** Both *ortho* **17a** and *para* isomers **17b** were isolated by RP-HPLC on a C18 symmetry shield column. **17a** (9.3 mg, 16%): t_R = 3.92 min; ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 300 MHz): δ = 8.53 (d, J = 1.5 Hz, 1H), 8.12 (dd, J = 8.1, 1.5 Hz, 1H), 7.75 (bs, 1H), 7.39 (d, J = 8.1 Hz, 2H), 7.32–6.96 (m, 11H), 6.83 (bt, J = 7.5 Hz, 1H), 6.76 (dd, J = 9.3 et 2.4 Hz, 2H), 6.64 (bs, 2H), 6.50 (d, J = 7.5 Hz, 1H), 5.25 (bdd, J = 11.0, 3.0 Hz, 1H), 4.46 (bs, 1H), 4.36 (bs, 2H), 4.29 (bt, J = 5.0 Hz, 2H), 3.96–3.80 (m, 8H), 3.69 (t, J = 4.8 Hz, 2H), 3.60–3.36 (m, 9H), 3.23 (bs, 3H), 2.95 (t, J = 5,1 Hz, 1H), 2.63–2.48 (bs, 4H), 2.3 (s, 3H), 2.0–1.80 (bs, 2H), 1.40–1.60 (bs, 2H), 1.25 ppm (m, 12H); ^{13}C NMR ($CDCl_3$/CD_3OD 9:1, 75 MHz): δ = 173.0, 172.6, 170,0, 169.4, 157.7, 156.1, 155.6, 148.5, 141.0, 140.6, 140.1, 138.6, 136.4, 135.9, 132.1, 131.5, 130.9, 130.7, 130.0, 129.7, 129.5, 128.3, 127.6, 127.5, 126.8, 125.8, 125.6, 124.2, 123.9, 118.8, 114.0, 95.9, 70.3, 70.1, 70.0, 69.5, 69.1, 50.5, 50.1, 48.0, 47.9, 42.9, 34.7, 31.4, 31.00, 25.2, 19.4, 12.4 ppm; RP-HPLC purity: >95%; **17b** (17 mg, 28%): t_R = 4.04 min; ^{1}H NMR ($CDCl_3$/CD_3OD 9:1, 400 MHz): δ = 8.63 (bs, 1H), 7.91 (bd, J = 8.1 Hz, 1H), 7.74 (bs, 1H), 7.38 (bs, 2H), 7.32–6.96 (m, 11H), 6.83 (b6t, J = 7.5 Hz, 1H), 6.71 (bd, J = 9.3, 2H), 6.60 (bs, 2H), 6.50 (d, J = 7.5 Hz, 1H), 5.14 (bd, J = 11.0 Hz, 1H), 4.48–4.24 (m, 5H), 3.84–3.68 (m, 10H), 3.58–3.36 (m, 9H), 3.23 (bs, 1H), 3.01 (bs, 1H), 2.91 (bt, J = 5,1 Hz, 1H), 2.89 (bs, 1H), 2.58–2.38 (m, 4H), 2.3 (s, 3H), 1.88–1.80 (bs, 2H), 1.58–1.44 (bs, 2H), 1.18 ppm (m, 12H); ^{13}C NMR ($CDCl_3$/CD_3OD 9:1, 100 MHz): δ = 176.4, 175.9, 173.4, 172.7, 172.13, 161.2, 160.7, 158.9, 150.1, 145.8, 144.0, 143.5, 142.0, 139.8, 139.3, 137.1, 136.2, 134.2, 134.1, 133.7, 132.9, 131.6, 131.0, 130.9, 130.2, 130.1, 159.0, 127.6, 12.2, 117.5, 117.0, 99.1, 73.7, 73.5, 73.0, 72.5, 53.9, 53.6, 50.0, 49.6, 49.2, 46.3, 38.1, 34.9, 34.3, 28.6, 22.7, 15.7, 11.8 ppm; RP-HPLC purity: >95%; HRMS calcd for $C_{67}H_{78}N_{10}O_{13}S_2$ 1295.5264; found: 1295.5269.

Radioligand binding assays: Binding assays were performed at 30°C using [^{3}H]AVP as the radioligand and Chinese hamster ovary (CHO) cell-membrane proteins (5 μg). Briefly, membranes prepared from CHO cells stably expressing the human V_2 AVP receptor were incubated in

50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris—HCl) pH 7.4, 5 mM $MgCl_2$, 1 mg mL^{-1} bovine serum albumin (BSA; binding buffer), and radiolabeled, displacing ligands for 30 min. The affinitiy K_d of [^{3}H]AVP to the V_2 human receptor has already been described earlier, and $K_d = (1.36 \pm 0.45)$ nM.[24] Affinities K_i of ligands **13, 17a**, and **17b** were determined by competition experiments using [^{3}H]AVP (1–2 nM) and varying the concentrations of the unlabeled ligands from 100 pM to 100 μM. The nonspecific binding was determined by adding unlabeled AVP (10 μM). Bound and free radioactive ligands were separated by filtration over Whatman GF/C filters presoaked in a 10 mg mL^{-1} BSA solution for 3–4 h. The ligand-binding data were analyzed by nonlinear least-squares regression using the computer program Ligand. All assays were performed in triplicate on at least three separate batches of cell membranes.

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