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PAPER

Trifunctional ^{99m}Tc based radiopharmaceuticals: metal-mediated conjugation of a peptide with a nucleus targeting intercalator†

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The development of molecular imaging agents with multiple functions has become a major trend in radiopharmaceutical chemistry. We present herein the syntheses of trifunctional compounds, combining an acridine orange (AO) based intercalator with a GRP receptor specific bombesin like peptide (BBN). Metal-mediated conjugation of these two functions *via* the [2 + 1] approach to the third function, the $[M(CO)_3]^+$ (M = ^{99m}Tc, Re) moiety, yielded the final trifunctional molecules. The strongly fluorescent acridine orange, a nuclear targeting agent, has been derivatised with 4-imidazolecarboxylate as a bidentate ligand and bombesin with an isonitrile group as a monodentate ligand. For cell and nuclear uptake studies, $[Re(L^1\text{-BBN})(L^2\text{-Ical})(CO)_3]$ type complexes were synthesized and characterized. For radiopharmaceutical purposes, the ^{99m}Tc analogues have been prepared in a stepwise synthesis. Fluorescence microscopy studies on PC-3 cells, bearing the BBN receptor, showed high and rapid uptake into the cytoplasm. For the bifunctional molecule, lacking the BBN peptide, no internalization was observed.

Introduction

The non-invasive visualization of molecular events *in vivo* and *in vitro* is referred to as molecular imaging.[1–3] The probes for imaging include *e.g.* PET and SPECT radiopharmaceuticals, MRI contrast agents and photoactive fluorescent compounds. Whereas the latter two modalities need a stimulus from outside (magnetic field or spectroscopic excitation) radiopharmaceuticals are active as such. Radiopharmaceuticals for molecular imaging comprise a targeting portion which strongly binds to, *e.g.*, receptors. Accumulation at the target site allows to visualize enhanced expression of cell receptors or metabolic processes such as glucose based rapid cell proliferation.[4] For cancer diagnosis, targets are typically cell bound peptide receptors which are often overexpressed in rapidly proliferating cells. In the near past, there are distinct trends to combine complementing imaging modalities, in particular fluorescent and radioactive probes. Such dual, triple or even quadruple imaging modalities, thus, combine targeting portions, fluorescent probes for *in vitro* optical visualization, radionuclides for *in vivo* imaging and *e.g.* magnetic nanoparticles for MRI.[5,6] In this context, radiopharmaceuticals may combine a luminescent and a radioactive probe. These probes may be separated from each other or be merged into one entity. Valliant *et al.* used a special type of isoquinoline based tridentate chelator which, upon coordination to the $[Re(CO)_3]^+$ moiety, switches luminescence to "on" and enables the visualization of the distribution of the compounds on the subcellular level.[7–9] Replacing the rhenium core with $[^{99m}Tc(CO)_3]^+$ yielded the homologues targeting radiopharmaceutical which can be used for *in vivo* imaging. Similar approaches with metal-induced luminescence have been reported by Parker *et al.* though without the extension to radionuclides of *e.g.* indium.[10]

In our own work, we aim at targeting the nucleus of specific cells with ^{99m}Tc or ^{188}Re radiopharmaceuticals. We showed *in vitro* that Auger and Koster–Kronig electrons emitted from ^{99m}Tc induced double strand breaks in DNA when localized in its intimate vicinity.[11,12] Thus, ^{99m}Tc could be used for targeted therapy and diagnosis in one single compound. Very recently, Reilly *et al.* showed that targeted ^{99m}Tc Auger electron therapy with the VEGF-2 K peptide is possible.[13] We showed that the combination of a nucleus targeting agent with a chelator bound to the $[Re(CO)_3]^+$ moiety accumulated in cell nuclei by confocal fluorescence microscopy.[14] Since these conjugates are not cell specific, we aim at the synthesis of trifunctional imaging compounds, comprising a receptor specific peptide, a nucleus targeting, luminescent part and the ^{99m}Tc complex. Conceptually, tri-functionality can be achieved *via* the metal complex to which the two modalities are separately coordinated or *via* a chelator to which both functions are covalently conjugated. The trifunctional metal complexes composed of the fluorescent organic dye pyrene, a nuclear localizing sequence (NLS) peptide and a tridentate chelator coordinated to the $[^{99m}Tc(CO)_3]^+$ moiety proved the concept but the syntheses of those conjugates lacked flexibility of intercalator and targeting vector variation (Scheme 1).[11,15]

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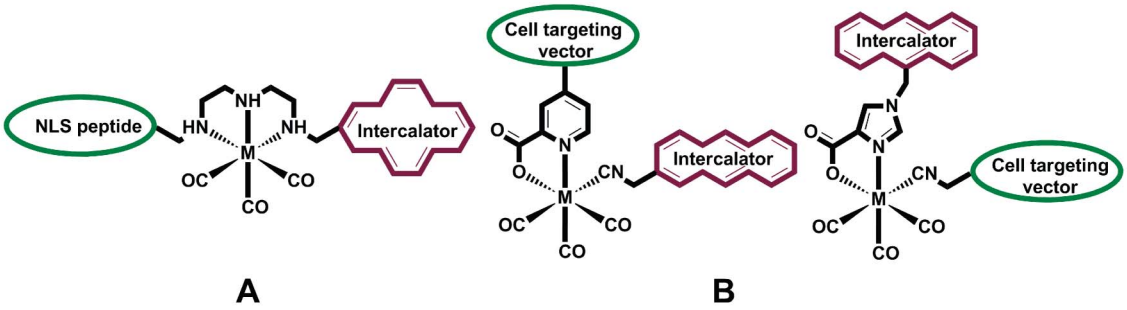
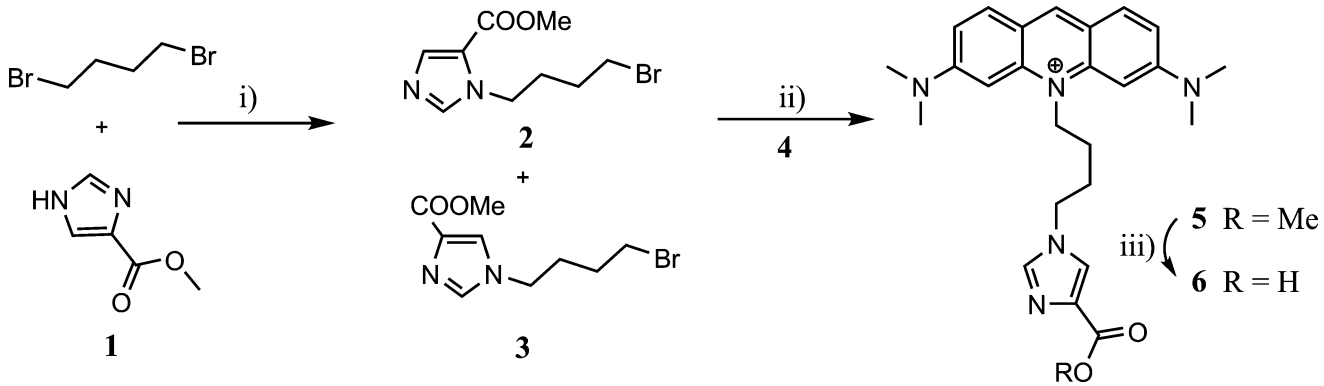


Scheme 1 Three approaches to trifunctional bioconjugates, (A) cell and nucleus targeting moieties are covalently linked to ligand,[11] and (B) metal-mediated conjugation of both modalities to the $[M(CO)_3]^+$ core *via* the [2 + 1] approach.



Scheme 2 Preparation of an acridine orange based, bifunctional ligand (i) K_2CO_3, DMF; (ii) **4**, toluene, ΔT; (iii) LiOH, H_2O–MeOH.

Since the cell receptor targeting vector should be cleaved in the intracellular space, conjugates based on the [2 + 1] approach fulfil the requirement of modular composition.[16] In this approach, the $[^{99m}Tc(CO)_3]^+$ core can be labeled with different targeting vectors and the intercalator moieties, allowing the variability necessary for drug development. The trifunctional conjugates presented by Agorastos *et al.* did not enter the cell nucleus, likely because the peptide was too strongly bound.[14] Thus, we hypothesized that a peptide bound *via* a monodentate ligand would facilitate its release into the intracellular space, leaving the nucleus targeting part behind. This approach is referred to as the inverted [2 + 1] approach. We present in this paper a synthetic study for the metal-mediated conjugation of an acridine orange based intercalator and a GRP receptor specific peptide according to the inverted [2 + 1] approach.

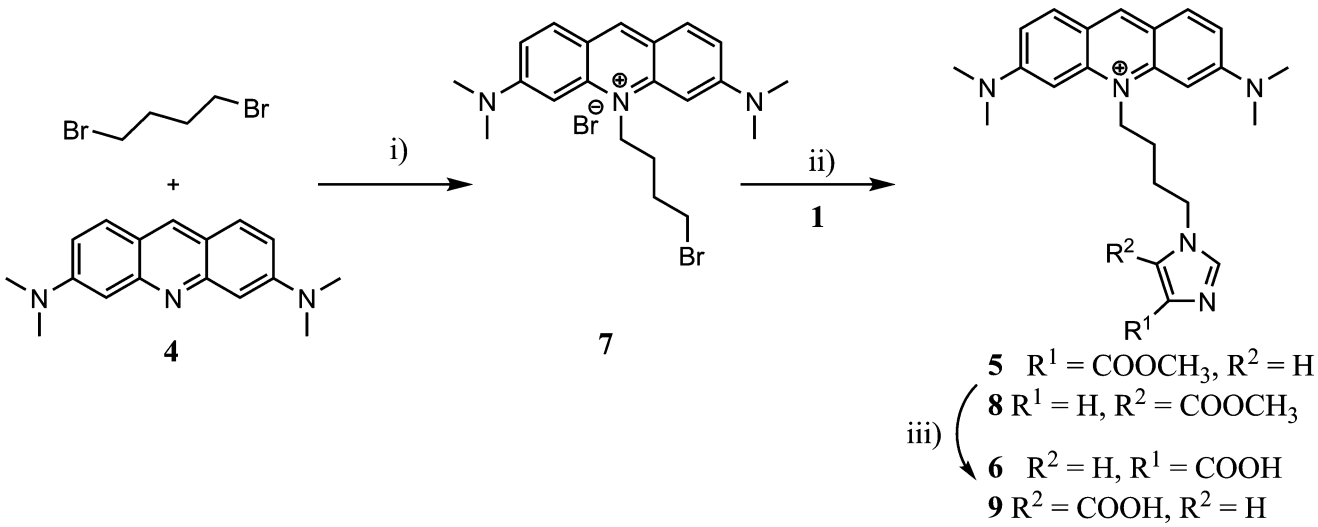
Results and discussion

Synthesis. According to the [2 + 1] approach, the nucleus targeting function should be bound to the $[^{99m}Tc(CO)_3]^+$ core *via* a bidentate chelator. Ideally, this chelator is mono-anionic in order to give a neutral complex $[Re(H_2O)(L^2\text{-Ical})(CO)_3]$. The water ligand is labile and can then be substituted by the monodentate ligand attached to the cell receptor targeting part. We found that isocyanides are excellent monodentate ligands. Due to its strongly fluorescent properties, we have selected acridine orange (AO) as the intercalator and methyl-4-imidazolecarboxylate **1** as the bidentate ligand. Compound **6** was prepared in three steps (including ester hydrolysis). In a first step, the alkylated imidazole **2** (and **3**) was prepared, followed by alkylation of the aromatic nitrogen in the free base of acridine orange (**4**) to yield **5**. Ester hydrolysis of **5** lead then to the free acid **6** (Scheme 2). Compound **6** comprises the nucleus targeting agent and the bifunctional chelator.

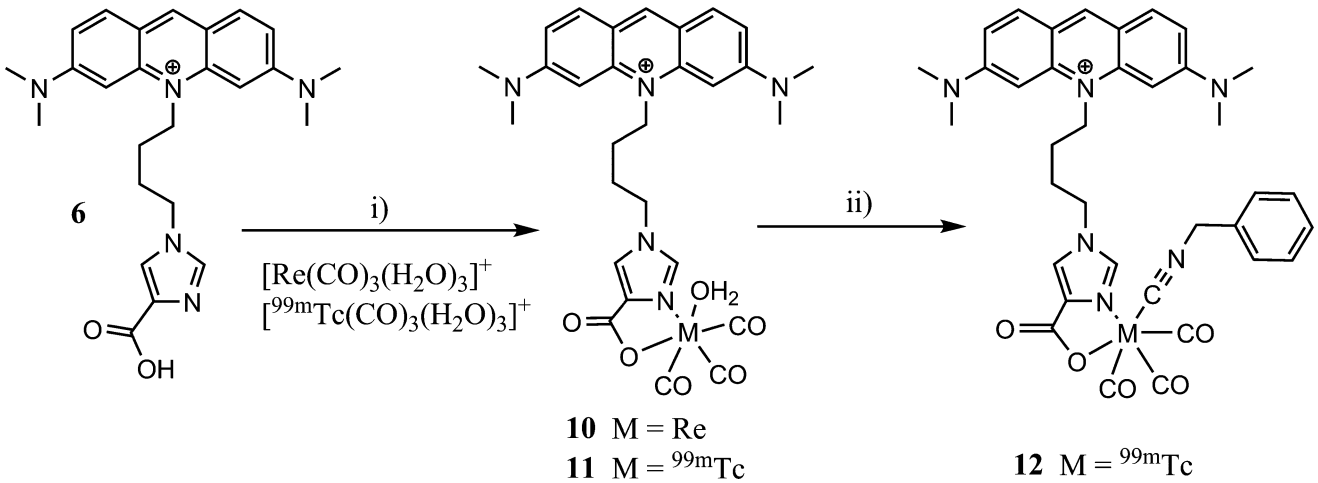
Due to the formation of polyalkylimidazolium side products, the quarternization yield was low. An alternative route for preparation of **6** was therefore used. Compound **7** was prepared by the reaction of AO free base with a large excess of 1,4-dibromobutane. The alkylation of methyl-4-imidazolecarboxylate **1** with **7** followed by hydrolysis of the ester group gave a mixture of the regioisomers **6** and **9** (1 : 1; Scheme 3).

These regioisomers could be separated but the mixture could also used in the next step since the bidentate ligand in **6** coordinates to $[Re(CO)_3]^+$ strongly enough to form a complex stable for subsequent purification. The reaction of **6** (eventually **6** + **9**) with $[Re(H_2O)_3(CO)_3]^+$ in a H_2O–MeOH mixture, afforded the rhenium complex **10** and the ^{99m}Tc homologue **11**, respectively (Scheme 4.).

The last step in the formation of the trifunctional complexes was the introduction of the targeting bombesin vectors **14** or **16**. Bombesin is a 14 amino acid neuropeptide with high affinity for the gastrin-releasing peptide (GRP) receptor, originally isolated from frog skin. Even modified bombesin derivatives keep their high GRP receptor affinity. Bombesin like peptides have been extensively studied for labeling with radionuclides[17,18] since the GRP receptor is overexpressed in many tumor cell lines. The truncated peptides **13** and **15** were prepared by standard solid phase synthesis (SPPS). The reaction of **13** or **15** with isocyanobutyric acid hydroxysuccinimidyl ester **21** gave the peptide-isonitrile derivatives **14** or **16** in quantitative yields. ESI-MS analysis confirmed the authenticities of the products. The reaction of **14** or **16** with complex **10** in acetonitrile/PBS gave the final product **17** or **19**



Scheme 3 (i) Toluene, ΔT; (ii) **1**, K_2CO_3, DMF; (iii) LiOH, H_2O–MeOH.



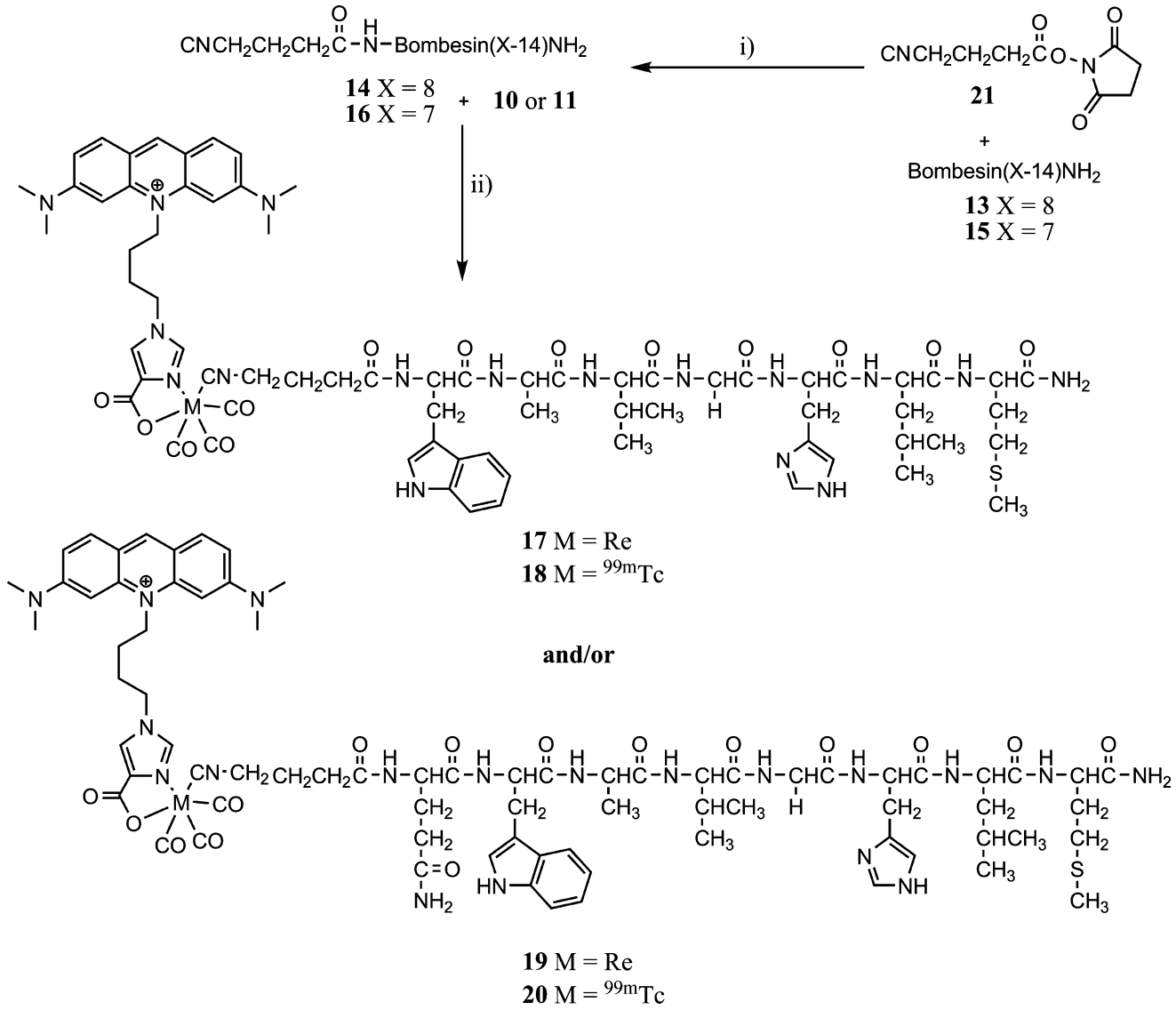
Scheme 4 Preparation of mixed ligand [2 + 1] complexes (i) MeOH–H_2O; (ii) BnNC, PBS/saline.

(Scheme 5). HPLC-MS analyses showed single peaks with the correct masses of 804.9 $[M+H]^{2+}$, 1608.7 $[M]^+$ and 868.9 $[M+H]^{2+}$, 1736.7 $[M]^+$, respectively.

Compounds **17** and **19** combine the nuclear-targeting molecules (AO) and the receptor-targeting portion (BBN), both independently bound to the Re or ^{99m}Tc (*vide infra*) center. The chemical stabilities of these conjugates were assessed by collecting samples of the compounds incubated in the cell cultivating medium after different periods of time. HPLC-MS analysis showed the conjugates to be stable over at least 24 h. No ligand exchange or decomposition was found and only the oxidation of methionine to the corresponding sulfoxide was observed.[19]

^{99m}Tc-labelling studies. The precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ was directly prepared from $[^{99m}TcO_4]^-$ as described.[20,21] The concentration of ^{99m}Tc was in the range of 1 nM. The solution was then buffered with 0.1 M phosphate buffer to pH 7.4. HPLC analyses with γ-detection were performed to quantify the conversion of the ^{99m}Tc. The [2 + 1] mixed ligand synthesis of complexes of the general type $[^{99m}Tc\ (L^1)(L^2)\ (CO)_3]$ consists of two consecutive steps. First, $[^{99m}Tc(H_2O)_3(CO)_3]^+$ reacted with the bidentate ligand L^2. Second, the monodentate ligand L^1 replaced the water ligand. In this way, coordination of **6** (20 μM) with $[^{99m}Tc(H_2O)_3(CO)_3]^+$ at 90 °C provided **11** in quantitative yield after 50 min. No coordination of a second ligand L^2 was observed. Subsequent addition of the ligand BnNC (40 μM) gave the complex **12** after 70 min at r.t. Compound **12** was slightly more lipophilic and, thus, a longer retention time, 17.8 min, was observed (Fig. 1, left). Analogous reaction of $[^{99m}Tc(H_2O)(\mathbf{6})(CO)_3]^+$ (**11**, dashed line right, retention time 25.0 min) with the peptide **14** at 80 °C gave $[^{99m}Tc(\mathbf{14})(\mathbf{6})(CO)_3]^+$ (**18**) in good yield after 60 min. Complex **18** was slightly more hydrophilic (Fig. 1, solid line right, retention time 24.5 min). The authenticity of **18** was confirmed by comparing the retention time of **18** with that of the Re analogue **17**. Comparable results were obtained for the reaction of the bombesin analogue **16** (BBN7-14) with **11**, yielding **20** with comparable retention time.

The availability of the trifunctional model compounds **17** and **19** and their radiolabeled analogues **18** and **20** serve as examples for the building block principle of combining individual targeting agents *via* binding to a metal core such as the $[M(CO)_3]^+$ moiety. In **20** for example, the nucleus targeting agent **6** is combined with the



Scheme 5 Preparation of the final, bombesin-based trifunctional compounds (i) DMF, Et_3N; (ii) MeCN/PBS or PBS/saline.

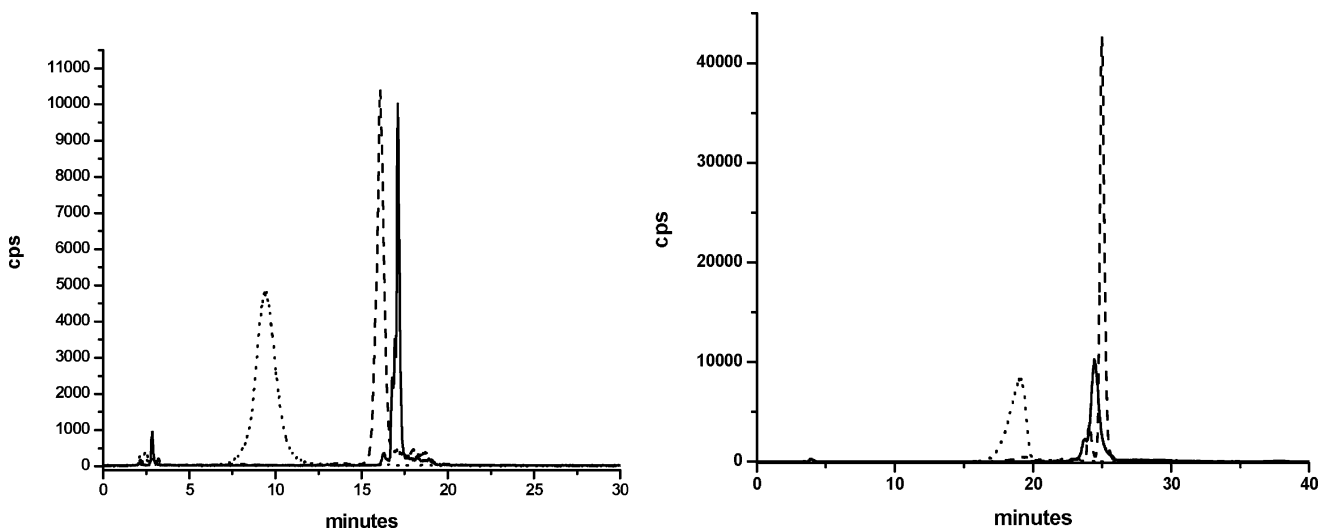


Fig. 1 Radioactive HPLC traces of $[^{99m}Tc(H_2O)(\mathbf{6})(CO)_3]^+$ **11** (dashed lines) and $[^{99m}Tc(H_2O)_3(CO)_3]^+$ (dotted lines). Left—formation of model complex $[^{99m}Tc(CN\text{-}Bn)(\mathbf{6})(CO)_3]^+$ **12** (solid line, Gradient C); Righ—formation of the trifunctional complex $[^{99m}Tc(\mathbf{15})(\mathbf{6})(CO)_3]^+$ **18** (solid line, Gradient D).

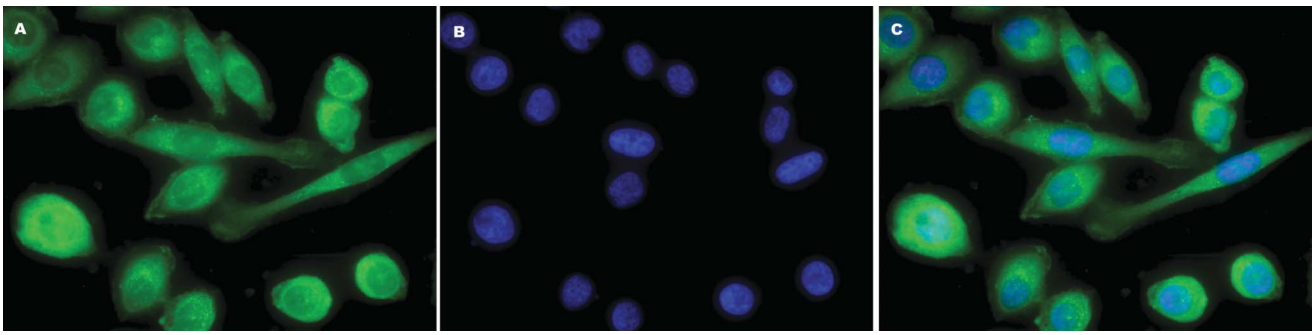


Fig. 2 Uptake of **19** by PC-3 cells, evaluated by fluorescence microscopy. Cells were incubated with 20 μM of **19** for 1 h followed by fixation of cells and nuclear staining by DAPI. (A) PC-3 cells were loaded with **19** in the cytoplasm (green). (B) Single channel images of the cell for DAPI staining (blue). (C) Merged image (overlay).

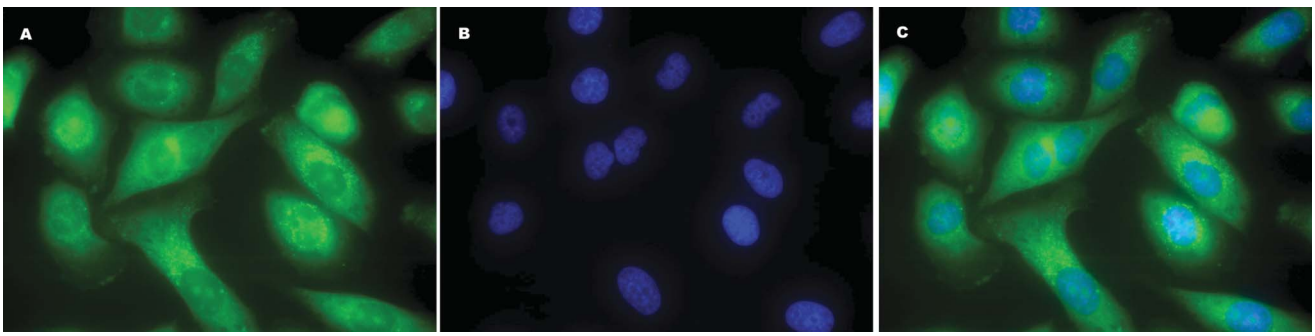


Fig. 3 Uptake of **19** by PC-3. Cells were incubated with complex **19** for 6 h according to the standard protocol. (A) PC-3 cells were loaded with **19** in the cytoplasm (green), punctuation like structures around the nucleus were observed. (B) DAPI staining (blue). (C) Merged image (overlay).

receptor specific peptide **15** both representing the two biological functions. The third function, the ^{99m}Tc core, serves in this system as the imaging or therapeutic portion. Each of these functions can be altered individually which allows for optimization of a particular agent without fully new syntheses. Admittedly, a two step synthesis to a trifunctional radiopharmaceutical is not ideal for routine application; however, it suffices the drug-finding concept in the first moment.[22]

Cell uptake studies. To study the cell uptake of the trifunctional compounds by confocal fluorescence microscopy, we selected the human prostate adenocarcinoma cell line PC-3 which does express the GRP receptor[23] and B16-BL6 mouse melanoma cell line with no expression of the GRP receptor. The PC-3 cell line was used for targeting studies with the model complex **10** and the BBN derivatives **17** and **19**. The uptake of complex **19** was studied as described in the experimental section. To localize the compounds in the cells and eventually in the nucleus, we stained the nuclei with DAPI (4′,6-diamidino-2-phenylindol). The PC-3 cells were exposed to **19** (20 μM) and, after 1 h, the distribution of **19** in the cells was detected by fluorescence microscopy (Fig. 2). Complex **19** accumulated in the cell and most of the fluorescence was localized in the cytoplasm.

Incubating the cells with **19** for 6 h revealed an interesting feature. The accumulation of **19** in the cells' cytoplasm produced a punctate like accumulation at distinct sites in the cell around the nuclei (Fig. 3). A similar heterogeneous distribution of peptides, derivatised with fluorescent ruthenium complexes, has recently been described.[24] In some cases, accumulation only took place in one particular region of the cell. Similar studies with **17** exhibited a similar time dependent behavior with **19** (see ESI†). It seems that the two compounds, regardless of their slightly different peptides, behave in a comparable way in the cells.

In contrast, in the absence of the receptor targeting vector (peptides BBN) such as in model complex **10**, almost no uptake was observed, even after extended incubation times (Fig. 4). Similar results were obtained for the pure acridine orange derivative **6** which carries no metal complex. This selective behavior of the full trifunctional cellular probes clearly underscores the requirement for a targeting vector in the trifunctional system and is in support of the cell specificity concept. The absence of uptake with **10** (or **6**) alone showed cell fluorescence to be due to internalization of the complete trifunctional conjugates and not just by eventual complex decomposition prior to the uptake.

To underline the cell specificity concept, similar studies were performed on a B16-BL6 mouse melanoma cell line which doesn't express the GRP receptor. While there was virtually no uptake of compound **19** in B16-BL6 cells, PC-3 cells were loaded with **19** in the cytoplasm, displaying the punctuate accumulation at specific sites as described before (Fig. 5). The uptake of **19** by the cells was significantly suppressed in B16-BL6 cells, indicating that this complex could not cross the cell membrane. The uptake of complex **19** by PC-3 cells was consistent with the presence of the GRP receptor, indicating a receptor-mediated uptake.

Conclusion

An efficient development of cell specific, nucleus targeting agents for molecular imaging and therapy requires a building block concept. In this work, we have applied the [2 + 1] concept for the

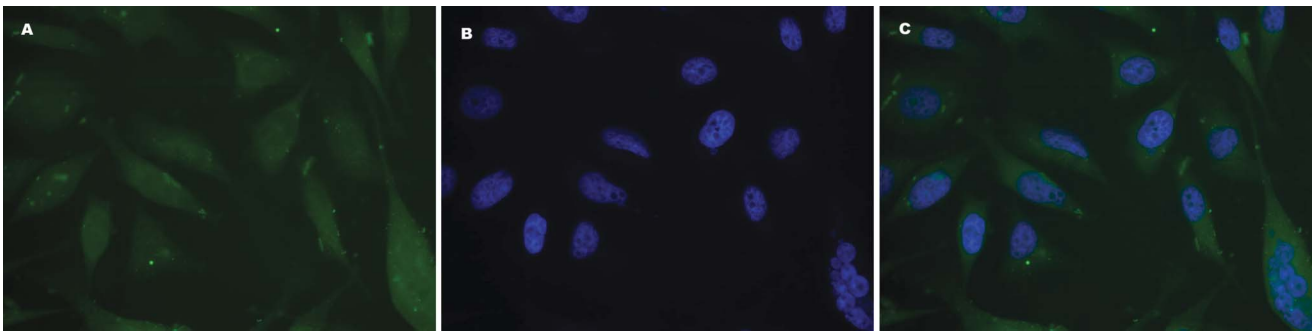


Fig. 4 Uptake of **10** by PC-3. Cells were incubated for 12 h according to the standard protocol. (A) Complex stain (green). (B) DAPI staining (blue). (C) Merged image (overlay).

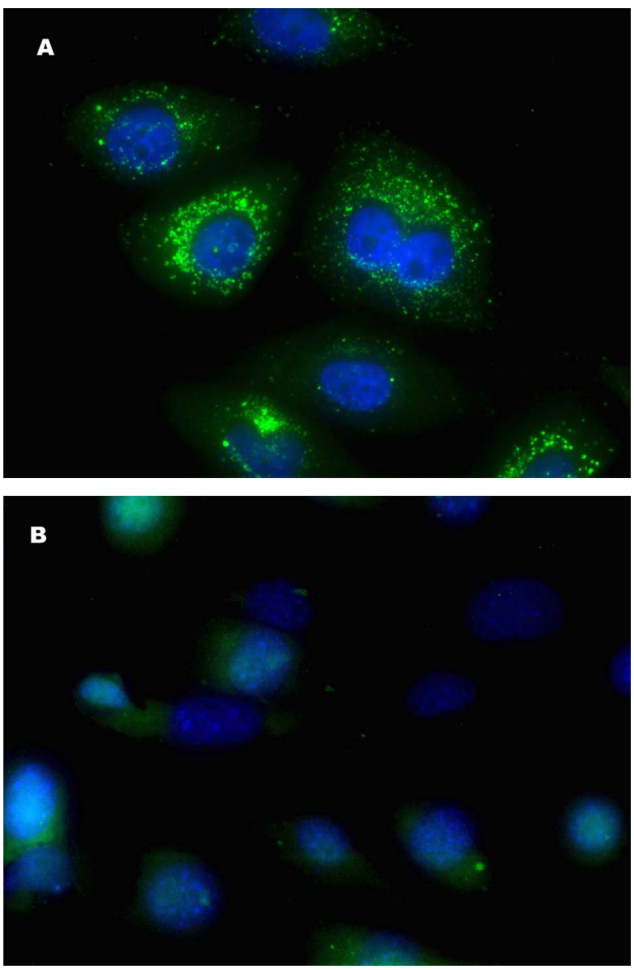


Fig. 5 Uptake of **19** by PC-3 and B16-BL6. Cells were incubated with complex **19** (20 μM) for 6 h, then the medium was changed and the cells were kept in the fresh medium for 24 h, fixed and stained according to the standard protocol. (A) PC-3 cells were loaded with **19** in the cytoplasm (green), punctuation around the nucleus (DAPI stain, blue) observed. (B) B16-F1 cells showed no uptake (background level green fluorescence).

formation of trifunctional radiopharmaceuticals. Fluorescence microscopy with Re compounds allows for localization of the compounds on the sub-cellular level whereas the analogous ^{99m}Tc compounds can be used for *in vivo* imaging. Although compounds **19** and **20** studied herein are not yet ideal (too little nucleus uptake), they underline the feasibility of the concept and reveal also the deficiencies of specific compounds. In particular, while the stable bifunctional complex did not penetrate the cell, the full trifunctional complexes were taken up into cells with the GRP receptor. The strength of the concept is the convenient alteration of any of its functions. Currently, detailed studies with ^{99m}Tc complexes bearing functionalities with improved nuclear uptake are being studied.

Experimental section

All solvents were of puriss or p.a. quality. The commercially available reagents were used as received without further purification. Isocyanobutyric acid hydroxysuccinimidyl ester (**21**) was a gift from Dr Hans-Jürgen Pietzsch, Forschungszentrum Rossendorf, Germany and is gratefully acknowledged. TLC on aluminium-based silica gel plates (silica gel 60 F_{254}) from Merck, Flash chromatography with Flashmaster Solo (Argonaut) with Merck silica gel 60 (0.040–0.063 mm). Samples were applied as almost saturated solutions in the appropriate solvent. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Gemini 200 and Bruker 400 spectrometers at 200 and 50 MHz, and 400 and 100 MHz respectively. The reported chemical shifts (in δ) are relative to the solvent protons and carbons. HPLC-MS (ESI) spectra were measured on a Bruker HCT spectrometer (Bruker) with Aquinity UPLC (Waters), using a Macherey-Nagel EC 250/3 Nucleodur C18 Gravity 5 μm. HPLC solvents were 0.1% formic acid (solvent A) and methanol (solvent B). The gradients used for analyses were: Gradient A: 0–5 min 90% A, 5–17 min 90–0% A, 17–25 min 0% A, 25–27 min 0–90% A, 27–35 min 90% A, flow 0.3 mL min^{-1}; Gradient B: 0–2 min 90% A, 2–12 min 90–0% A, 12–17 min 0% A, 17–18 min 0–90% A, 18–25 min 90% A, flow 0.25 mL min^{-1}. HPLC analyses were performed on a Merck L7000 system using a Macherey-Nagel EC 250/3 Nucleodur C18 Gravity 5 μm for radioactive compounds. HPLC solvents: 0.1% trifluoroacetic acid (solvent A) and MeOH or MeCN HPLC grade (solvent B). Gradient C (MeOH): 0–5 min 90% A, 5–15 min 90–0% A, 15–20 min 0% A, 20–22 min 0–90% A, 22–30 min 90% A. Gradient D (MeCN): 0–5 min 90% A, 5–20 min 90–60% A, 20–23 min 60% A, 23–28 min 0% A, 28–33 min 0% A, 33–34 min 0–90% A, 34–40 min 90% A. Flow rates: 0.5 mL min^{-1}, detection with a γ-detector for the radiolabeled compounds. Preparative HPLC was performed on a Varian Pro Star system by using either a Macherey-Nagel VP 250/21 Nucleodur C18 Gravity 5 μm or a Macherey-Nagel

VP 250/40 Nucleosil 100–7 C18 column with flow rates of 12 mL min^{-1} and 40 mL min^{-1}, respectively. The solvents were 0.1% trifluoroacetic acid (solvent A) and methanol or acetonitrile (solvent B). The exact mass measurements were obtained using peak top or centroid depending on the peak shape for each analyte (see ESI†). The elementary analyses for some compounds were not performed due to the various contents of trifluoroacetate after purification with the preparative HPLC. Thus, to be consistent, the M.W. and concentrations of the compounds were calculated without counter-ions (*e.g.* as drawn in the schemes). If not specified in the scheme, the counter-ions are trifluoroacetates.

Cell culture and complex uptake. Human prostate cancer cells, PC-3 were incubated in F12 K medium supplemented with 10% fetal bovine serum. B16-BL6 mouse melanoma cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. For microscopy, cells were cultured overnight on 4-chamber slides (Nunc) in which *ca.* 50 000 cells were plated. The next day, cells were washed and fresh medium containing complex **19**, **17**, **10** or **6** (20 μM) was added. Cells were incubated with the complex for 1–24 h. After loading with a complex, cells were washed with phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde for 10 min at r.t. After three washings with PBS, cells were incubated in 1 mg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining for 10 min at r.t. Cells were washed three times with phosphate-buffered saline (PBS), chambers from the slides were detached and cells were covered with coverslips in Prolong Mounting medium (Invitrogen). In order to get well resolved images for the cell specificity studies (B16-BL6 *vs.* PC-3), the loaded cells were kept for 24 h in the fresh medium prior to fixation. Slides were evaluated on Zeiss Axiovert 200 m with the corresponding fluorescence filters for DAPI (λ_{ex} = 360 nm, λ_{em} = 420 nm) and acridine orange (λ_{ex} = 496 nm, λ_{em} = 525 nm).

Methyl-1-(4-bromobutyl)-1*H*-imidazole-4-carboxylate (2). A suspension of methyl-4-imidazolecarboxylate (**1**) (200 mg, 1.58 mmol) and K_2CO_3 (700 mg, 5 mmol, dry) in DMF (10 mL, dry) was stirred at r.t. under N_2. After 10 min, 1,4-dibromobutane (1.9 ml, 3.4 g, 15.8 mmol) was added and the mixture stirred and heated to 50 °C under N_2. After 16 h, the solvent was removed under HV. The residue was dissolved in 15 mL of CH_2Cl_2, filtered over Celite, and dried again. Purification by flash chromatography (gradient, hexane to EtOAc) afforded two main fractions. The second fraction contained 130 mg of 1,4 regioisomer **2** (32%) as a light yellow oil. The compound was immediately used in the next step. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.62 (s, 1H), 7.49 (s, 1H), 4.02 (t, ^3J = 7.0 Hz, 2H), 3.88 (s, 3H), 3.40 (t, ^3J = 6.4 Hz, 2H), 2.02–1.95 (m, 2H), 1.90–1.80 (m, 2H). MS (ESI) *m/z* (%) 261.4 (100), 263.4 (98) $[M + H]^+$. Calcd for $C_9H_{14}BrN_2O_2^+$: 261.0 (100.0%), 263.0 (98.2%). Elemental analysis (%) calcd for $C_9H_{13}BrN_2O_2$: C 41.40, H 5.02, N 10.73; found: C 41.75, H 4.81, N 10.94. TLC R_f 0.13 (EtOAc). The first fraction contained 113 mg of 1,5 regioisomer **3** (27%) as a light yellow oil. For analyses see the ESI.†

3,6-Bis(dimethylamino)-10-(4-(4-(methoxycarbonyl)-1*H*-imidazol-1-yl)butyl)acridinium (5). A solution of **2** (120 mg, 0.46 mmol) and acridine orange free base **4** (60 mg, 0.22 mmol) in toluene (10 mL) was stirred under N_2, for 24 h at 120 °C. The formed precipitate was filtered and washed with hexane and dried *in vacuo*. Purification of the crude product by flash chromatography (gradient, CH_2Cl_2 to CH_2Cl_2–MeOH–isopropylamine 100 : 5 : 1) afforded two fractions. The second fraction contained 7 mg of **5** (7%) as an orange powder. ^{1}H NMR (CD_3OD, 400 MHz): δ 8.55 (s, 1H), 7.90–7.74 (m, 4H), 7.53–7.45 (dd, ^3J = 9.3 Hz, ^4J = 2.0 Hz, 2H), 6.50 (s, 2H), 4.64 (t, ^3J = 8.0 Hz, 2H), 4.14 (t, ^3J = 6.6 Hz, 2H), 3.75 (s, 3H), 3.20 (s, 12H), 2.12–2.02 (m, 2H), 1.92–1.80 (m, 2H). HPLC-MS (ESI, Gradient A) t_R: 15.7 min; *m/z*: 446.3 (100) $[M]^+$. Calcd for $C_{26}H_{32}N_5O_2^+$: 446.3 (100%). The first fraction contained 3,6-bis(dimethylamino)-10-methylacridinium. Analyses were similar to the literature.[25]

10-(4-(4-Carboxy-1*H*-imidazol-1-yl)butyl)-3,6-bis(dimethylamino)acridinium (6). **5** (5 mg, 0.011 mmol) was dissolved in MeOH (1 mL) and a solution of LiOH in water (0.5 mL, 1 M) was added to the mixture. The reaction was stirred and heated to 50 °C for 12 h. After the neutralization with HCl (0.5 M), the solvent was removed *in vacuo*. The crude product was purified by preparative HPLC to give **6** as an orange powder in a yield of (4.6 mg, 95%). ^{1}H NMR (CD_3OD, 400 MHz): δ 8.67 (bs, 1H), 8.64 (s, 1H), 8.10 (bs, 1H), 7.88 (d, ^3J = 9.2 Hz, 2H), 7.24 (d, ^3J = 8.9 Hz, 2H), 6.62 (s, 2H), 4.74 (bt, ^3J = 8 Hz, 2H), 4.29 (bt, ^3J = 6.5 Hz, 2H), 3.27 (s, 12H), 2.26–2.09 (m, 2H), 2.06–1.91 (m, 2H). HPLC-MS (ESI, Gradient A) t_R: 14.5 min; *m/z*: 432.2 (100) $[M]^+$. Calcd for $C_{25}H_{30}N_5O_2^+$: 432.2 (100%). The mixture of **6** and **9** was obtained according to the preparation **2/3** using compound **7** instead of 1,4-dibromobutane, followed by hydrolysis as for **6**. The regioisomers were separated using preparative HPLC to afford **6** and **9**. For analyses of **9** see the ESI.†

10-(4-Bromobutyl)-3,6-bis(dimethylamino)acridinium bromide (7). Acridine orange free base **4** (50 mg, 0.19 mmol) was dissolved in toluene (dry, 5 mL) and 1,4-bromobutane (0.5 ml, 912 mg, 4.22 mmol). The mixture was heated to reflux. After 24 h, the reaction mixture was diluted with toluene (15 mL) and the precipitate filtered. The crude product was washed with toluene and toluene with few drops of NH_4OH. The residue was dissolved in CH_2Cl_2 and the orange solution was diluted with hexane until product precipitated. The solution was basified with a few drops of NH_4OH, stirred for 10 min at r.t. and filtered. Finally, the precipitate was washed with hexane and dried under HV to give **7** (70 mg, 77%) as a dark orange powder. ^{1}H NMR (CD_3OD + $CDCl_3$, 200 MHz): δ 8.61 (s, 1H), 7.88 (d, ^3J = 9.2 Hz, 2H), 7.14 (d, ^3J = 9.2 Hz, 2H), 6.77 (bs, 2H), 4.81 (bt, ^3J = 8 Hz, 2H), 3.72 (t, ^3J = 5.8 Hz, 2H), 3.35 (s, 12H), 2.45–2.00 (m, 4H). HPLC-MS (ESI, Gradient A) t_R: 16.6 min; ESI-MS: *m/z*: 402.1 (100), 400.1 (98) $[M\text{-}Br]^+$. Calcd for $C_{21}H_{27}BrN_3^+$: 402.1 (100.0%), 400.1 (99.9%). Elemental analysis (%) calcd for $C_{21}H_{27}Br_2N_3$: C 52.41, H 5.65, N 8.73; found: C 53.05, H 5.55, N 8.47.

$[Re(H_2O)(6)(CO)_3]^+$ (10). To a solution of **6** (4 mg, 0.0092 mmol) in CH_3OH–H_2O (1 mL, 1 : 1) $[NEt_4]_2[ReBr_3(CO)_3]$ (8 mg, 0.01 mmol) was added. The mixture was stirred at r.t. under N_2 for 4 h. A precipitate was filtered, washed with water and hexane, and dried in desiccator. The crude product was purified by preparative HPLC to give **10** as orange powder in a yield of 5 mg (61%). ^{1}H NMR (CD_3CN, 400 MHz): δ 8.55 (s, 1H), 7.89 (s, 1H), 7.83 (d, ^3J = 9.4 Hz, 2H), 7.47 (s, 1H), 7.16 (dd, ^3J = 9.3 Hz, ^4J = 2.1 Hz, 2H), 6.51 (s, 2H), 4.59 (bt, ^3J = 8 Hz, 2H), 4.12 (t, ^3J = 6.8 Hz, 2H), 3.22 (s, 12H), 2.09 (m, 2H), 1.90 (m, 2H). HPLC-MS (ESI, Gradient B) t_R: 12.2 min; *m/z*: 702.3 (100) $[M\text{-}H_2O]^+$, 700.3

(57) $[M\text{-}H_2O]^+$. Calcd for $C_{28}H_{29}N_5O_5Re^+$: 702.2 (100.0%), 700.2 (57.6%).

Bombesins WAVGHLM (13) and QWAVGHLM (15). Truncated bombesins **13** (sequence: WAVGHLM) or **15** (sequence: QWAVGHLM) were synthesized SPPS according to a procedure previously reported.[14] The products were then purified by reverse phase HPLC on a preparative C18 column (Macherey-Nagel VP 250/40 Nucleosil 100–7 C18 column) using a gradient of 5–55% acetonitrile in water (+ 0.1% TFA) over 45 min and a flow of 40 mL min^{-1}. Fractions with the peptides were lyophilized; pure peptides were kept under N_2 in the freezer at –25 °C. **13** and **15** were obtained as white solids. **13**: HPLC-MS (ESI, Gradient A) t_R: 13.3 min; positive mode *m/z*: 812.5 (100) $[M+H]^+$, 406.7 (56) $[M+2H]^{2+}$; negative mode *m/z*: 810.5 (56) $[M-H]^-$, 924.5 (100) $[M+TFA]^-$. Calcd for $C_{38}H_{58}N_{11}O_7S^+$: 812.4 (100.0%). **15**: HPLC-MS (ESI, Gradient B) t_R: 10.6 min; positive mode *m/z*: 940.7 (100) $[M+H]^+$, 470.9 (98) $[M+2H]^{2+}$; negative mode *m/z*: 938.7 (100) $[M-H]^-$. Calcd for $C_{43}H_{66}N_{13}O_9S^+$: 940.5 (100.0%).

4-Isocyanobutyryl-bombesins (14) and (16). The bombesin **13** (23 mg, 0.02 mmol) was dissolved in DMF (dry, 1 mL) with Et_3N (14 µl, 10.1 mg, 0.1 mmol). The mixture was stirred at r.t. under N_2. After 15 min, isocyanobutyric acid hydroxysuccinimidyl ester **21** (4.5 mg, 0.022 mmol) was added and the mixture was stirred at r.t. for 6 h. Solvents were removed *in vacuo*. The product **14** was pure enough for use in the next step without further purification. HPLC-MS (ESI, Gradient B) t_R: 11.4 min; positive mode *m/z*: 907.5 (100) $[M+H]^+$; negative mode *m/z*: 905.5 (100) $[M-H]^-$, 1019.7 (20) $[M+TFA]^-$. Calcd for $C_{43}H_{63}N_{12}O_8S^+$: 907.5 (100.0%). The bombesin derivative **16** was prepared by a similar manner from bombesin **15**. HPLC-MS (ESI, Gradient B) t_R: 12.0 min; positive mode *m/z*: 1035.7 (100) $[M+H]^+$; negative mode *m/z*: 1033.8 (100) $[M-H]^-$. Calcd for $C_{48}H_{71}N_{14}O_{10}S^+$: 1035.5 (100.0%).

$[Re(12)(6)(CO)_3]^+$ (17). Compounds **14** (5 mg, 0.0055 mmol) and **10** (2 mg, 0.0028 mmol) were dissolved in an acetonitrile/PBS mixture (1 mL, 1 : 1, pH 7.4). The solution was stirred for 8 h at 70 °C. The MS-ESI and HPLC-MS analysis confirmed the formation of desired product **17**. The solvent was removed *in vacuo* and the crude product was purified by preparative HPLC to give **17** (0.5 mg, 11%) as an orange powder. MS (ESI) *m/z* (%): 1608.8 (100). HPLC-MS (ESI, Gradient B) t_R: 11.7 min; *m/z*: 804.9 (100) $[M+H]^{2+}$, 1608.7 (3) $[M]^+$, 1609.7 (25) $[M]^+$. Calcd for $C_{71}H_{91}N_{17}O_{13}ReS^+$: 1608.6 (100.0%), 1609.6 (75.5%).

$[Re(12)(6)(CO)_3]^+$ (19). Complex **19** was prepared by a similar manner as **17**, using **16** (7 mg, 0.0067 mmol) and **10** (4 mg, 0.0057 mmol). The crude product was purified by preparative HPLC to give **19** (1 mg, 11%) as an orange powder. HPLC-MS (ESI, Gradient B) t_R: 12.2 min; *m/z*: 868.9 (100) $[M+H]^{2+}$, 579.6 (21) $[M+2H]^{3+}$. Calcd for $C_{76}H_{99}N_{19}O_{15}ReS^+$: 1736.7 (100.0%), 1737.7 (79.8%).

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