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Labeling of the Neuropeptide Enkephalin with Functionalized Tris(pyrazolyl)borate Complexes: Solid-Phase Synthesis and Characterization of *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ and *p*-[Enk-OH]$COC_6H_4Tp^{Me}Re(CO)_3$

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Bioconjugates of the neuropeptide Enkephalin (Enk = –Tyr–Gly–Gly–Phe–Leu–) labeled with organotransition metal tris-pyrazolylborate (Tp) complexes are reported. *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ and *p*-[Enk-OH]$COC_6H_4Tp^{Me}$-$Re(CO)_3$ have been synthesized by solid-phase peptide synthesis (SPPS), purified by RP-HPLC, and characterized by ESI-mass spectrometry and ^{1}H NMR and IR spectroscopies. *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ and *p*-[Enk-OH]COC_6H_4-$Tp^{Me}Re(CO)_3$ constitute the first examples of Tp complexes employed in SPPS.

Biomolecules labeled with inorganic coordination complexes have been employed in a variety of biochemical and medicinal applications ranging from heavy atom probes and sensors to radiochemical imaging and drugs.[1] For such applications, organometallic complexes have found use but to a limited extent because of their inherent tendency to decompose in the presence of water and oxygen. Thus, for the investigation of organometallics in biologically and medicinally related applications, the metal coordination environment must be quite robust, particularly in aqueous solution. Very recently there has been a resurgence of interest in bioorganometallic chemistry.[2] One particular area that has received significant attention is organometallic bioconjugates, compounds in which an organometallic complex is covalently bonded to a biomolecule (e.g., peptides, DNA, RNA, drugs).[3] We have been interested in the synthesis, characterization, and application of organometallic bioconjugates as infrared and electrochemical markers and for biomedical studies.[4] Specifically, we have focused on bioconjugates in which the biomolecule and metal complex are covalently linked through the ligand framework, as opposed to a direct bond between the metal and biomolecule. We have synthesized several examples of such bioconjugates by using a functionalized metal complex in a solid-phase peptide synthesis (SPPS) protocol.[5] As such, the organometallic complexes we employ in this manner must be stable under the conditions used for SPPS which are not suitable for the handling of most organometallic compounds. Thus, there remains interest in

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the discovery of highly robust organometallics which may be employed in SPPS.

Transition metal complexes have been employed in a wide array of biochemical and medicinal applications. One outstanding example are complexes of Group 7 which have been used extensively in radiochemistry such as the clinically employed Cardiolite,[6] a hexa-isonitrile complex of ^{99m}Tc(I). Complexes of radioactive rhenium (^{186}Re, ^{188}Re) have also been investigated[7] and have potential in nuclear medicine therapies, for example bone pain relief.[8] Notably, carbonyl complexes of Group 7 have been extensively studied quite recently, spurred on by Alberto's discovery of a facile method for generating $[Tc(CO)_3(H_2O)_3]^+$.[9] A variety of transition metal carbonyls have also been employed as infrared markers as a result of their strong absorptions in the range of ca. 1750−2120,[10] a region which is normally blank in most biomolecules, and as CO-releasing therapeutics.[1b,11] Moreover, third-row transition elements have been used for many years as heavy atom probes in protein crystallography and transmission electron microscopy.[1b]

We have recently reported that the σ-organoplatinum(IV) tris-pyrazolylborate (Tp) complex, *p*-$(HO_2C)C_6H_4TpPtMe_3$, undergoes coupling to amino acid monomers in homogeneous solution.[12] Herein we extend our studies on coupling between transition metal [*p*-$(HO_2C)C_6H_4Tp$] complexes and biomolecules and report the solid-phase synthesis, isolation, and characterization of the labeled Enkephalin (Enk = −Tyr−Gly−Gly−Phe−Leu−) derivatives, *p*-[Enk-OH]-$COC_6H_4TpPtMe_3$ and *p*-[Enk-OH]$COC_6H_4Tp^{Me}Re(CO)_3$, the first examples of Tp bioconjugates made using SPPS.

The first attempt to employ a Tp complex in SPPS focused on the attachment of *p*-$(HO_2C)C_6H_4TpPtMe_3$ to the neuropeptide Enk, a natural ligand to the opiate receptor. We have previously labeled Enk with metallocenes and organomolybdenum complexes.[13] We use Enk in this work only as a model peptide because it is a relatively small and easy-to-handle peptide, not for its biological function. At this early stage, no effort is made to improve biological stability or receptor binding by conjugation to the metal complex. Initially, Enk was synthesized according to standard Fmoc-SPPS methods[14] on a Wang resin, a polystyrene matrix functionalized with *p*-benzyloxybenzyl alcohol linker groups. The Fmoc group was removed from the Tyr fragment, *p*-$(HO_2C)C_6H_4TpPtMe_3$ was activated with TBTU and HOBT and coupled to the resin-bound Enk, and the mixture was subsequently treated with 95% TFA to cleave the product from the resin. Analysis of the resultant product was inconsistent with the formulation as *p*-[Enk-OH]COC_6H_4-$TpPtMe_3$ from the conspicuous absence of the [$PtMe_3$] group. For example, the ^{1}H NMR spectrum of the product contains signals for Enk and a Tp ligand but lacks the signature signal for a [$TpPtMe_3$] complex which consists of a singlet with Pt satellites (^{195}Pt, $I = 1/2$, 33.83% abundance, J_{Pt-H} = ca. 70 Hz).[15] Furthermore, in the mass spectrum (ESI positive mode) the peak for {*p*-[Enk-OH]$COC_6H_4TpPtMe_3 + H^+$}at $m/z = 1111.19$ (exact mass = 1111.43) is only 5% as intense as the largest cluster of peaks observed at $m/z = 875-870$, which are suggestive of the {*p*-[Enk-OH]COC_6H_4Tp} fragment (exact mass = 870.39). Taken together, the spectral evidence implied that the coupling reaction did in fact occur but that a high percentage of the [$TpPtMe_3$] group was partially degraded during the cleavage reaction. Thus, it was necessary to employ a resin which releases bound peptides under milder conditions. ^{1}H NMR experiments in DMF-d_7 with mesitylene as an internal standard demonstrated that the phenylalanine conjugate *p*-[Bu^{t}O-Phe]$COC_6H_4TpPtMe_3$ is stable for at least 5 weeks in the presence of ca. 20% v/v TFA. The "2-chloro Trityl resin" (2-ClTrt), a 1% DVB copolystyrene matrix functionalized with 2-chlorotrityl linker groups, is known to release bound peptides upon mildly acidic treatment with acetic acid, 0.5% TFA, or hexafluoroisopropanol.[16] Accordingly, it seemed feasible to synthesize and isolate the desired product by using the 2-Cl-Trt-resin as the solid support for SPPS. Indeed, coupling of *p*-$(HO_2C)C_6H_4$-$TpPtMe_3$ to 2-Cl-Trt-resin-bound Enk, deprotection, and cleavage with 10% v/v TFA in DMF[17] and purification by RP-HPLC gave *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ (Scheme 1).

In the ESI mass spectrum (negative mode) of the product, the largest peak is observed at $m/z = 1109.38$ which corresponds nearly perfectly with [*p*-[Enk-OH]COC_6H_4-$TpPtMe_3 - H^+$] (exact mass= 1109.41). Furthermore, in the ^{1}H NMR spectrum (methanol-d_4), the [$PtMe_3$] group (J_{Pt-H} = 70 Hz) and the two diastereotopic methyl groups of the [Leu] fragment, moieties which are located at opposite ends of the labeled oligopeptide, appear in a 1:1 molar ratio, as shown in Figure 1. These data cumulatively provide support for the product formulation as *p*-[Enk-OH]$COC_6H_4TpPtMe_3$.

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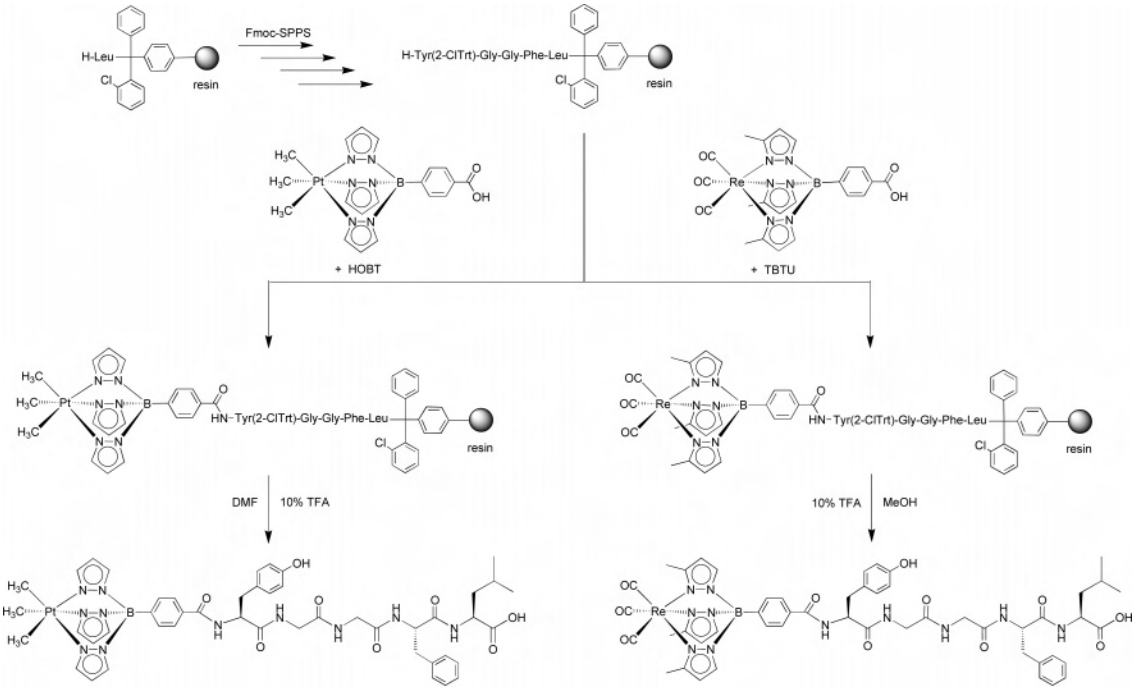
Scheme 1. SPPS Coupling of *p*-$(HO_2C)C_6H_4TpPtMe_3$ and *p*-$(HO_2C)C_6H_4Tp^{Me}Re(CO)_3$ to Enkephalin.



In a similar fashion to *p*-[Bu^{t}O-Phe]$COC_6H_4TpPtMe_3$, the rhenium tricarbonyl complex *p*-$(HO_2C)C_6H_4Tp^{Me}Re(CO)_3$ was shown to be stable in DMF-d_7 containing ca. 20% v/v TFA for 2 days. Accordingly, the bioconjugate *p*-[Enk-OH]-$COC_6H_4Tp^{Me}Re(CO)_3$ was also synthesized and isolated, as shown in Scheme 1. The product was characterized by ^{1}H NMR, ESI (negative mode) mass spectrometry, which showed one major peak at $m/z = 1182.35$, consistent with [*p*-[Enk-OH]$COC_6H_4Tp^{Me}Re(CO)_3 - H^+$] (exact mass = 1182.37), and IR spectroscopy which had two distinct, very strong metal carbonyl absorbtions at 2020 and 1890 cm^{-1} and strong amide I and II absorbtions from the peptide moiety at 1653 and 1516 cm^{-1}, as shown in Figure 2.[18] These data support the product formulation as *p*-[Enk-OH]$COC_6H_4Tp^{Me}$-$Re(CO)_3$.

Both *p*-[Enk-OH]$C_6H_4TpPtMe_3$ and *p*-[Enk-OH]COC_6H_4-$Tp^{Me}Re(CO)_3$ were tested for antiproliferative activity using crystal violet and resazurin assays. The results showed no toxicity for either bioconjugate complex at concentrations up to 1 mM. For these bioconjugates, the lack of toxicity renders them potentially useful for in vivo IR, heavy atom labeling or use as CO-releasing molecules (CORMs).

In summary, we have reported the solid-phase synthesis, isolation, and characterization of the labeled Enk derivatives *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ and *p*-[Enk OH]$COC_6H_4Tp^{Me}$-$Re(CO)_3$. Considering that Tp is one of the most well studied ligands in inorganic chemistry[19,20] it is notable that *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ and *p*-[Enk-OH]$COC_6H_4Tp^{Me}Re(CO)_3$ are the first examples of Tp bioconjugates synthesized by

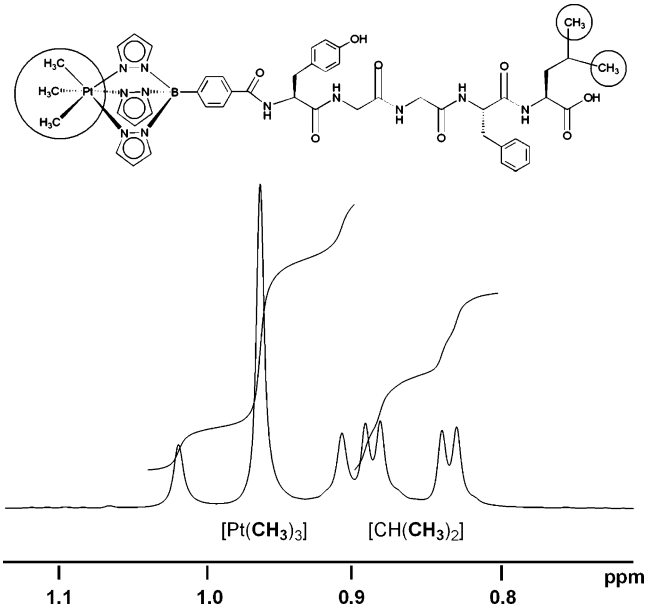


Figure 1. Selected region of the ^{1}H NMR spectrum of *p*-[Enk-OH]-$COC_6H_4TpPtMe_3$.

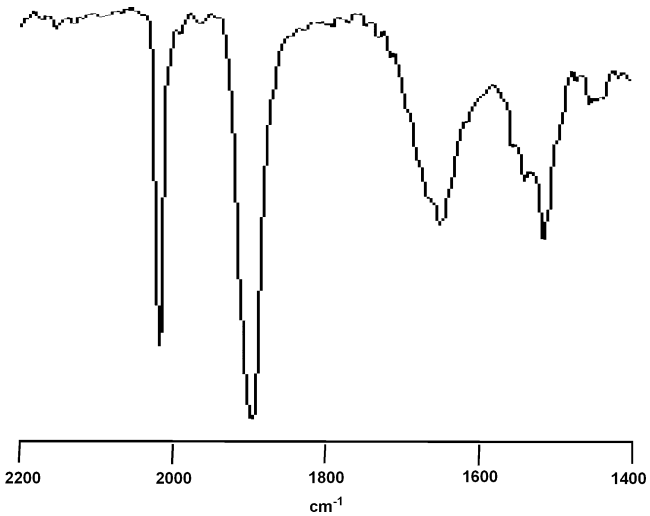


Figure 2. Selected region of the IR spectrum of *p*-[Enk-OH]COC_6H_4-$Tp^{Me}Re(CO)_3$.

SPPS methods thereby demonstrating the potential for employing Tp complexes in this type of methodology.

Experimental Section

All reagents and solvents were purchased from commercial sources and used as received. H-Leu-2-chlorotrityl resin (H-Leu-2-ClTrt) and all amino acid derivatives were purchased from Novabiochem. The Fmoc-Leu-Wang resin, HOBT monohydrate, and TBTU were purchased from Iris Biotech (Germany). HOBT monohydrate is 1-hydroxybenzotriazole monohydrate, TBTU is 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, DIPEA is *N*,*N*,*N*-diisopropylethylamine. Electrospray ionization mass spectra (ESI-MS) were recorded on Finnigan Thermo LCQ and Bruker Esquire 6000 spectrometers. The ^{1}H NMR spectra were recorded at ambient temperature on Bruker DPX 200, DPX 250 and DRX 600 spectrometers. The NMR chemical shifts (δ) are reported in ppm relative to the residual proton chemical shifts of the deuterated solvent set relative to external TMS. Coupling constants (J) are quoted in Hz. The test of stability in the presence of TFA was carried out by making a solution of *p*-[(Bu^{t}O-Phe]-$COC_6H_4TpPtMe_3$ in 500 μL of DMF-d_7 with 5 μL of mesitylene added as an internal standard. The ^{1}H NMR spectrum was recorded and the ratio of *p*-[(Bu^{t}O-Phe-CO]$C_6H_4TpPtMe_3$ to mesitylenewas determined by comparison of the intergrals from the [$PtMe_3$] and mesitylene-methyl signals. TFA was then added to the solution to a level of 20% v/v in TFA and the ^{1}H spectrum was recorded immediately and subsequently at random intervals up to ca. 5 weeks. Comparison of the [$PtMe_3$] and mesitylene-methyl intergrals showed a contant ratio of *p*-[Bu^{t}O-Phe]$COC_6H_4TpPtMe_3$ to mesitylene over the monitored period. The same procedure was followed to determine the stability of *p*-$(HO_2C)C_6H_4Tp^{Me}Re(CO)_3$ using the $Tp^{Me}-CH_3$ and Pz−CH resonances for comparison with mesitylene. The analytical and preparative HPLC were both carried out on a Varian Prostar instrument using a RP Varian Dynamax analytical column (C18 microsorb 60 Å, diameter 4.5 mm, 250 mm length) with water and acetonitrile, both containing 0.1% v/v TFA, as eluents using a linear gradient of 20−100% acetonitrile for 30 min at a flow rate of 1 mL/min. Infrared spectra were recorded on Perkin-Elmer 1720X and Bruker Tensor 27 spectrometers. $(HO_2C)C_6H_4TpPtMe_3$ was made according to the published procedure.[12]

***p*-$BrC_6H_4Tp^{Me}Re(CO)_3$.** Toluene (ca. 20 mL) was added to a stirred mixture of *p*-BrC_6H_4 $Tp^{Me}Tl$ (0.67 g, 1.09 mmol) and bromopentacarbonylrhenium (0.45 g, 1.10 mmol), and the reaction mixture was heated at 90 °C overnight with the system open to an oil bubbler. The volatile components of the reaction mixture were removed under reduced pressure, the residue extracted into hot toluene (90 °C, ca. 40 mL), and filtered to give a pale yellow filtrate. The volatile components of the filtrate were removed under reduced pressure leaving *p*-BrC_6H_4 $Tp^{Me}Re(CO)_3$ as an off-white solid (0.59 g, 80%). $C_{21}H_{19}BBr_3N_6O_3Re$ Calcd: C, 37.07%, H, 2.81%, N, 12.35%; Found: C, 37.07%, H, 2.85%, N, 12.33%. ν (KBr) 1914 and 2013 cm^{-1} ν_{CO} of $Re(CO)_3$. δ_H (250 MHz, $CDCl_3$) 2.59 (9H, s), 6.05 (3H, t, J = 2.4), 7.40(3H, d, J = 2.4), 7.65 (2H, d, J = 8.4), 7.76 (2H, d, J = 8.4); δ_C (62.5 MHz, $CDCl_3$) note: one (C) resonance, presumed to be the B-*C* carbon, is not oberved. 16.1 (CH_3), 106.1 (CH), 123.1 (C), 131.3 (CH), 136.1 (CH), 136.7(CH), 154.2 (C).

***p*-$(HO_2C)C_6H_4Tp^{Me}Re(CO)_3$.** ^{n}BuLi (0.55 mL, 2.5 M soln in hexane, 1.38 mmol) was added dropwise over a few minutes to a cold (ca. −80 °C) solution of *p*-BrC_6H_4 $Tp^{Me}Re(CO)_3$ (0.85 g, 1.25 mmol)) in THF (ca. 15 mL), and the mixture was stirred for ca. 90 min cold. Excess solid dry ice (ca. 2 g) was added, the mixture was removed from the cold bath and stirred for ca. 60 min, and then the volatile components were removed under reduced pressure leaving a green solid which was washed with ether (3 × 10 mL) and dried under vacuum. On the benchtop open to air, the solid was slurried with aqueous HCl (ca. 15 mL, 2%) for 30 min then filtered and dried to give *p*-$(HO_2C)C_6H_4Tp^{Me}Re(CO)_3$ as a white solid (0.48 g, 51%). $C_{22}H_{20}BN_6O_5Re$ Calcd: C, 40.94%; H, 3.12%; N, 13.02%; Found: C, 40.63%; H, 3.14%; N, 12.76%. IR (KBr) 1690 cm^{-1} ν_{CO} of CO_2H, 1916 and 2015 cm^{-1} ν_{CO} of $Re(CO)_3$, (neat solid) 1892 and 2017 cm^{-1}; δ_H (200 MHz, $CDCl_3$) 2.60 (9H, s), 6.06 (3H, t, J = 2.3), 7.41 (3H, d, J = 2.3), 8.03 (2H, d, J = 8.4), 8.23 (2H, d, J = 8.4); δ_C (62.5 MHz, CDCl3), note: two (C) resonances, presumed to be the B-*C* and Pz-C3 carbons, is not oberved. 16.1 (CH_3), 106.2 (CH), 129.5 (CH), 135.2 (CH), 136.1-(CH), 154.3 (C), 170.6 (CO_2H).

***p*-[Enk-OH]$COC_6H_4TpPtMe_3$.** 2-Chlorotrityl-resin-bound {-(2Cl-Trt)Tyr-GlyGlyPheLeu}, (2Cl-Trt)Enk-2-Cl-TrtRES, was synthesized by standard SPPS methods.[14] The metal complex was coupled to the resin-bound peptide as detailed here. *p*-$(HO_2C)C_6H_4$-$TpPtMe_3$ (0.80 g, 0.14 mmol), TBTU (0.043 g, 0.13 mmol), HOBT monohydrate (0.021 g, 0.14 mmol), and DIPEA (50 μL) were combined in 1 mL of DMF, mixed vigorously, and let stand for 5 min. The activated carboxylic acid solution thus prepared was shaken with (2Cl-Trt)Enk-2-Cl-TrtRES (0.077 g, 0.49 mmol/g loading) for ca. 20 h. After coupling *p*-$(HO_2C)C_6H_4TpPtMe_3$ to the oligopeptide, the reaction mixture was filtered, the resin was washed with DMF (3 × 1 mL), dichloromethane (3 × 1 mL), and methanol (3 × 1 mL), and then dried under reduced pressure for ca. 30 min. The product was cleaved from the resin by treatment with a solution of trifluroacetic acid (1 mL, 10% v/v in DMF) for ca. 90 min. The reaction mixture containing the crude *p*-[Enk-OH]-$COC_6H_4TpPtMe_3$ product was filtered, the resin was extracted with methanol (4 × 1 mL), and the combined filtrates were concentrated under reduced pressure to a volume of ca. 200−300 μL to which cold diethylether (ca. 10 mL, −70 °C) was added, which produced a hazy white mixture. The mixture was then centrifuged and the solvents decanted to collect a white and colorless oily solid. The decanted solvent mixture was again concentrated under reduced pressure to ca. 100−200 μL, and the cold ether (ca. 10 mL, −70 °C), centrifuge, and decant cycle was repeated to provide another crop of white solid. The white solids collected were dried under reduced pressure, combined, and dissolved in a minimum volume of methanol, and the crude product was purified by reverse-phase HPLC giving *p*-[Enk-OH]$COC_6H_4TpPtMe_3$ as a white powder (10 mg, 26% based upon the resin loading of 0.49 mmol/g). $C_{47}H_{58}BN_{11}O_8Pt$ calcd exact mass = 1140.40, found MS (ESI, negative mode) 1109.38, [M − H$^+$]. δ_H (600 MHz, methanol-d_4) note: the resonances from one α-CH and two β-CH_2 groups are obscured by the solvent peaks. Assignments are based, in part, upon comparison to literature data on Enk: *Eur. J. Biochem.* **1990**, *192*, 433. 8.06 (2H, CH of [BrC_6H_4], d, J = 7.8), 7.96 (2H, CH of [BrC_6H_4], d, J = 7.8), 7.68 (3H, CH of Pz, J = 2.2), 7.59 (3H, CH of Pz, J = 2.2), 7.26−7.21 (5H, C_6H_5 of Phe), 7.15 (2H, CH of Tyr *p*-phenol, d, J = 8.0), 6.73 (2H, CH of Tyr *p*-phenol, d, J = 8.0), 6.28 (3H, CH of Pz, J = 2.2), 4.65 (1H, α-CH, dd, J = 9.0, 5.5), 4.39 (1H, α-CH, dd, J = 9.0, 5.5), 3.95−3.77 (4H, 2 × α-CH_2, m), 3.08 (2H, β-CH_2, dd, J = 13.8, 9.0), 2.96 (2H, β-CH_2, dd, J = 13.8, 9.0), 1.70−1.57 (3H, α-CH and β-CH_2, m) 0.97 (9H, "s"

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(20) See for example Zn−enzyme models: (a) Parkin, G. *Chem. Rev.* **2004**, *104*, 600−767. (b) Parkin, G. *Chem. Comm.* **2001**, 1971−1985.

with Pt satellites, $J_{Pt-H} = 70$), 0.88 (3H, Tyr-CH_3, d, $J = 6.3$), 0.83 (3H, Tyr-CH_3, d, $J = 6.3$).

***p*-[Enk-OH]$COC_6H_4Tp^{Me}Re(CO)_3$.** This compound was prepared and isolated in a fashion analogous to that of *p*-[Enk-OH]-COC_6H_4TpPtMe using (2Cl-Trt)Enk-2-Cl-Trt resin (0.077 g, 0.49 mmol/g loading), *p*-$(HO_2C)C_6H_4$ $Tp^{Me}Re(CO)_3$ (0.10 g, 0.154 mmol), TBTU (0.048 g, 0.151 mmol), HOBT monohydrate (0.022 g, 0.147 mmol), and DIPEA (44 μL). The cleavage was carried out with TFA (1 mL, 10% v/v in MeOH) for 2 h. Yield after RP-HPLC was 25 mg, 55%. $C_{50}H_{55}BN_{11}O_{11}Re$ calcd exact mass = 1183.37, found MS (ESI, negative mode) 1182.25, [M − H^+]. IR (neat solid) ν_{CO} of $Re(CO)_3$ 1890 and 2020, 1516 (amide II), and 1653 cm^{-1} (amide I); δ_H (600 MHz, methanol-d_4) note: the resonances from one α-CH and two β-CH_2 groups are obscured by the solvent peaks. Assignments are based, in part, upon comparison to literature data on Enk: *Eur. J. Biochem.* **1990**, *192*, 433. 7.95 (2H, CH of [BrC_6H_4], d, $J = 8.3$), 7.92 (2H, CH of [BrC_6H_4], d, $J = 8.3$), 7.48 (3H, CH of Pz^{Me}, $J = 2.2$), 7.26−7.12 (5H, C_6H_5 of Phe; 2H, CH of Tyr *p*-phenol, d, $J = 8.3$), 6.73 (2H, CH of Tyr *p*-phenol, d, $J = 8.3$), 6.14 (3H, CH of Pz^{Me}, $J = 2.2$), 4.61 (1H, α-CH, dd, $J = 9.7$, 4.5), 4.27 (1H, α-CH, dd, $J = 9.7$, 4.5), 3.95−3.77 (4H, 2 × α-CH_2, m), 3.07 (2H, β-CH_2, dd, $J = 14.1$, 8.6), 2.93 (2H, β-CH_2, dd, $J = 14.1$, 8.6), 2.58 (9H, CH_3 of Pz^{Me}, s) 1.66−1.51 (3H, α-CH and β-CH_2, m), 0.88 (3H, Tyr-CH_3, d, $J = 5.9$), 0.83 (3H, Tyr-CH_3,d, $J = 5.9$).

Cell Culture Conditions. The human HT29 colon cancer cell line was obtained from the Institute of Pharmacy of the Free University of Berlin. The cell line was cultured in McCoy Medium (Sigma, Germany) supplemented with 10% Fetal Calf Serum (Gibco, Germany), 2 mM l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated in 75 cm^2 cell culture flasks at 37 °C and in a humidified atmosphere with 5% CO_2. The cell lines were passaged weekly using 0.05% trypsin with 0.02% EDTA (Gibco, Germany).

Cytotoxicity Assays. Two assays, the crystal violet assay[21] and the resazurin assay[22] (both Sigma), were performed with the HT29 cell line on 96-well microtiter plates. A 40 000 cells/mL suspension in culture medium (99 μL) was plated into each well and incubated for 24 h at 37 °C and 5% CO_2. By addition of 1 μL of a stock solution (100, 20, and 5 mM) of the respective compound in methanol the desired concentrations (1, 200, and 50 μM) in 1% methanol was reached. After 48 h of incubation, the cells first underwent the resazurin and subsequently the crystal violet assay. Cells were washed two times with colorless RPMI 1640 medium (PAA Laboratories, Germany) without Phenol red and without Fetal Calf Serum. Next, 90 μL of colorless RPMI 1640 and 10 μL of resazurin were added to each well. Absorbance was directly measured at 600 nm, and the measurement was repeated after 2 h of incubation. Activity of mitochondrial dehydrogenases was determined by the decrease in absorbance.

Next, the cell biomass was determined by a crystal violet assay. The medium was removed, and cells were fixed with 4% paraformaldehyde in PBS. Cells were washed with PBS (phosphate buffered saline) and afterward with 0.1% Triton-100 (Sigma) in PBS.

Cells were then stained with a 0.04% crystal violet solution and subsequently washed four times with distilled water. Crystal violet was extracted by 96% ethanol, and the absorbance was determined at 570 nm. Values were corrected for the absorbance at the start of substance incubation. Vehicle controls (methanol), negative controls (culture medium), and positive controls (Cis-Platin (Sigma)) were also determined.

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