RESEARCH PAPER

Artificial DNA: PNA & XNA 4:1, 11–18; January/February/March 2013; © 2013 Landes Bioscience

Di-heterometalation of thiol-functionalized peptide nucleic acids

Tanmaya Joshi,[1] Malay Patra,[1] Leone Spiccia[2] and Gilles Gasser[1,*]

[1]Institute of Inorganic Chemistry; University of Zurich; Zurich, Switzerland; [2]ARC Centre of Excellence for Electromaterials Science and School of Chemistry; Monash University; Clayton, VIC Australia

Keywords: peptide nucleic acids, hetero-metalation, ruthenium, cymantrene, thioether, organometallics

Abbreviations: Cys, cysteine; DIPEA, diisopropylethylamine; ESI-MS, electrospray ionization mass spectrometry; Fmoc, fluorenylmethoxycarbonyl; Gly, glycine; HPLC, high performance liquid chromatography; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate; IR, infrared; LC-MS, liquid chromatography-mass spectrometry; MALDI-TOF, matrix assisted laser desorption/ionization time of flight; PNA, peptide nucleic acid; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trt, trityl

As a proof-of-principle, two hetero-bimetallic PNA oligomers containing a ruthenium(II) polypyridyl and a cyclopentadienyl manganese tricarbonyl complex have been prepared by serial combination of solid-phase peptide coupling and in-solution thiol chemistry. Solid-phase *N*-terminus attachment of Ru(II)-polypyridyl carboxylic acid derivative, C1, onto the thiol-functionalized PNA backbone (H-a-a-g-t-c-t-g-c-linker-cys-NH_2) has been performed by standard peptide coupling method. As two parallel approaches, the strong affinity of thiols for maleimide and haloacetyl group has been exploited for subsequent post-SPPS addition of cymantrene-based organometallic cores, C2 and C3. Michael-like addition and thioether ligation of thiol functionalized PNA1 (H-gly-a-a-g-t-c-t-g-c-linker-cys-NH_2) and PNA2 (*C1*-a-a-g-t-c-t-g-c-linker-cys-NH_2) to cymantrene maleimide and chloroacetyl derivatives, C2 and C3, respectively, has been performed. The synthesized ruthenium(II)-cymantrenyl PNA oligomers have been characterized by mass spectrometry (ESI-MS) and IR spectroscopy. The distinct Mn-*CO* vibrational IR stretches, between 1,924–2,074 cm^{-1}, have been used as markers to confirm the presence of cymantrenyl units in the PNA sequences and the purity of the HPLC-purified PNA thioethers assessed using LC-MS.

Introduction

Peptide Nucleic Acids (PNAs),[1] a robust class of artificial nucleic acids, have for several years now been recognized as molecules with enormous diagnostic and therapeutic potential.[2-6] PNAs, in general, are prepared by conventional solid-phase peptide synthesis (SPPS).[7] This seems to be simple and uncomplicated nowadays because traditional PNA sequencing can be easily achieved on an automated synthesizer. Since their invention, consistent efforts have been made to widen the window for diagnostic applications from this artificial oligomeric construct. In this pursuit, both metallic as well as non-metallic detection labels have been coupled to PNAs.[8-13] Adding functionalities to PNAs, however, have always imposed challenges for chemists. To be effective, such approaches should specifically produce the desired product and, more importantly, show compatibility with the functional groups found in the available PNA monomers as well as the inserted labels. As far as synthetic challenges are concerned, the magnitude further increases if the desired modifications are metal-based. The growing interest in the application of metal-containing PNAs[11,14] has led to several different approaches to prepare metal-containing PNA constructs. Leaving aside the formation of metal-containing PNA by metal complexation of a ligand-containing PNA,[15-27] such intrasequence covalent metallo-labeling of the PNA sequence can be achieved using either appropriately designed metal-containing PNA monomers[28-34] or through classical amide coupling between the carboxylic acid derivatives of the metal-complexes and the reactive terminal amino group on PNA backbone.[35-39] In recent times, "click chemistry" has also become a popular tool for introducing organometallic fragments into PNA-like monomers, PNA oligomers and peptides.[40-44] Much to our surprise, however, multi-metallic labeling of PNAs has been consistently overlooked despite the potential applications of these bioconjugate in diagnostics, therapeutics and biosensing.[45]

Over the past few years, research in our groups has focused on the design and evaluation of metal-PNA bioconjugates for multimodal biosensing, nuclear medicine, molecular and cell biology applications.[46-51] Toward this end, targeting the development of robust direct (solid-phase) and indirect (solution phase) ways for the preparation of multi-metallic PNA conjugates, we recently reported the first di-hetero-organometallic-containing PNA

*Correspondence to: Gilles Gasser; Email: gilles.gasser@aci.uzh.ch
Submitted: 12/23/12; Revised: 02/14/13; Accepted: 02/15/13
http://dx.doi.org/10.4161/adna.24019

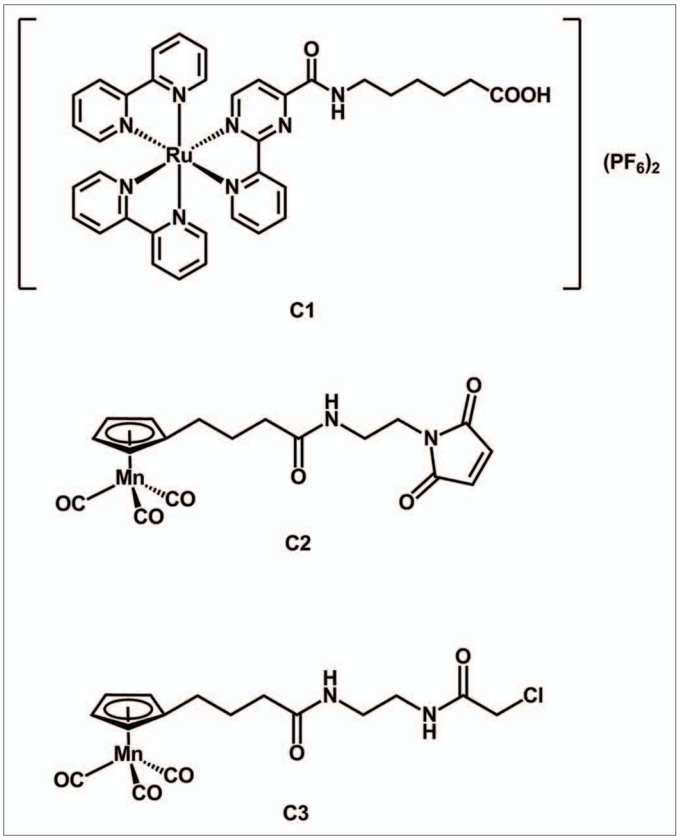


Figure 1. Structure of Ru(II) and $CpMn(CO)_3$ derivatives (C1–C3).

oligomers, synthesized using sequential "click chemistry" and peptide coupling reactions.[45] Adding to these recent endeavors to devise strategies for including multiple metallo-inserts in PNA oligomers, in this work, we demonstrate the use of powerful and versatile thiol chemistry for the convergent synthesis of hetero-bimetallic PNA oligomers. Thioether ligation and Michael type addition of thiol-functionalized PNA sequences are applied in combination with conventional peptide coupling, to demonstrate an extremely effective strategy for obtaining hetero-metallic PNA conjugates. For this proof-of-principle study, a carboxylic acid derivative of a substitutionally inert Ru(II) polypyridyl complex, C1, and maleimide or chloroacetyl cymantrenyl derivatives, C2 and C3, are employed as metallolabels on a 8-mer thiol-functionalized model PNA oligomer, PNA1 (**Fig. 1**).

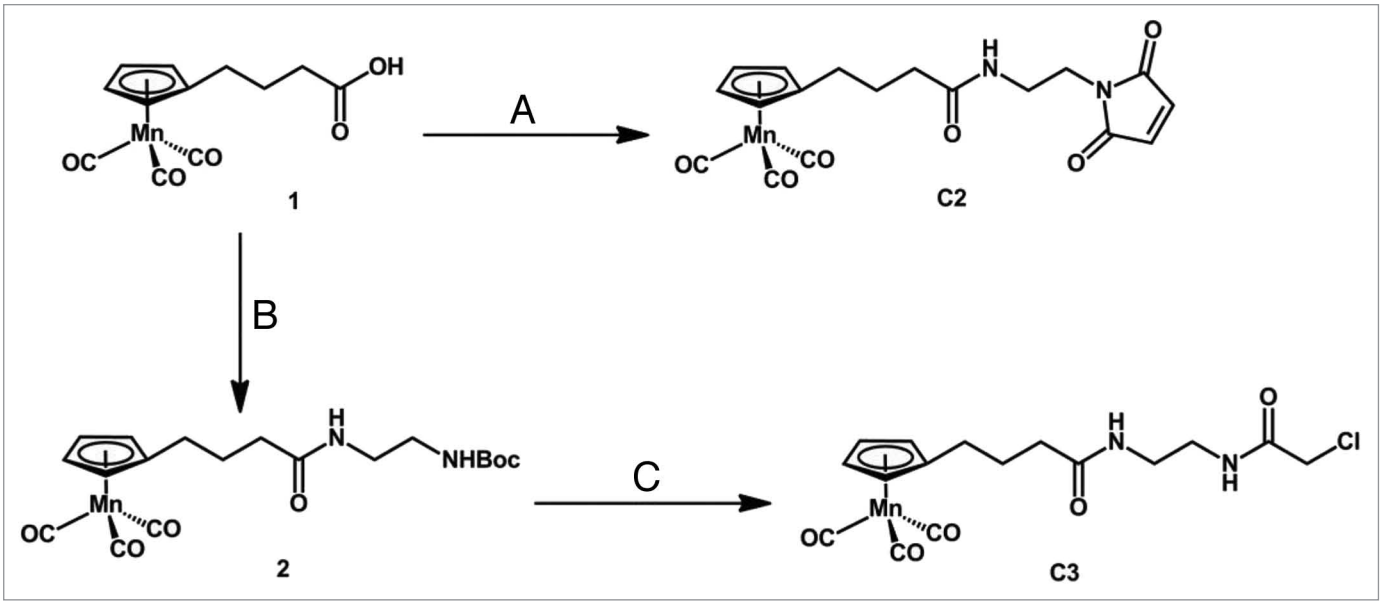
Results and Discussion

Synthesis of cymantrenyl derivatives. As shown in **Scheme 1**, the maleimide-functionalized cymantrene derivative, C2, was obtained from condensation between its free carboxylic precursor, 4-cymantrenylbutyric acid (1), and the trifluoroacetate salt of *N*-(2-aminoethyl)maleimide, in presence of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HATU) and triethylamine. The desired product was successfully isolated as a yellow solid in 61% yield. The ^{1}H NMR for C2 showed signals at 6.73 ppm for the vinylic protons from the maleimide fragment, and from the protons on the cyclopentadienyl moiety at ca. 4.64 ppm. Additional signals were found in the ^{1}H NMR between 1.81–3.70 ppm, ascribable to alkyl CH_2 protons, along with the ^{13}C NMR showing the characteristic C≡O signal at 225.2 ppm. The IR spectrum showed strong C≡O vibrational stretches at 2010 and 1905 cm^{-1} and C=O stretching vibration bands at 1703 and 1635 cm^{-1}. Furthermore, peak at *m/z* = 435 for $[M+Na]^+$ ion in ESI-MS confirmed the successful conjugation of the maleimide fragment to the cymantrenyl backbone in C2.

The synthesis of compound 2 was performed in an analogous manner to C2. Employing HATU-mediated peptide coupling, mono Boc-protected ethylenediamine was successfully attached to the manganese tricarbonyl derivative 1 (**Scheme 1**). The ^{1}H and ^{13}C NMR spectra for 2 showed signals originating from the *tert*-butyl functionality at 1.45 ppm as well as 28.6 and 80.1 ppm, respectively. The ^{13}C NMR spectrum also showed a $Mn(CO)_3$ core resonance at 225.4 ppm. Strong vibrational bands for C≡O (1921 and 2016 cm^{-1}) and C=O (1643 and 1693 cm^{-1}) stretches in the IR spectrum and the $[M+Na]^+$ peak at *m/z* = 455.1 in ESI-MS verified the successful isolation of 2. The acetyl chloride derivative, C3, was prepared by trifluoroacetic acid (TFA)-mediated deprotection of the *tert*-butyl group and subsequent acetylation of the obtained free amine with chloroacetyl chloride (**Scheme 1**). As expected, the ^{1}H and ^{13}C NMR spectra showed the disappearance of *tert*-butyl resonances along with the appearance of an additional singlet from the acetyl (-COCH_2Cl) unit at 4.04 ppm. Strong carbonyl stretches (C≡O and C=O) between 1635–2012 cm^{-1} in the IR spectrum in conjunction with the $[M+H]^+$ ion peak (*m/z* = 408.5) seen in the ESI-MS confirmed the identity of C3.

Synthesis of thiol-functionalized PNA oligomers. The thiol-functionalized model PNA oligomer, PNA1, was prepared on the TentaGel S RAM Cys(Trt)Fmoc resin, following manual solid-phase peptide synthesis protocols (**Scheme 2**).[40,47,49,50] Sequential coupling of the Fmoc-Bhoc protected PNA monomers, cleavage of the non-metalated oligomer from the resin using TFA/water/triisopropylsilane (TIS) 95/2.5/2.5 (v/v/v), followed by RP-HPLC purification gave the desired oligomer, PNA1 (refer to Experimental Section for more details). Conclusions on the identity and purity of PNA1 were derived from the respective ESI-MS, MALDI-TOF spectra and LC-MS traces. Peaks at *m/z* = 499.1 $[M+5H]^{5+}$, 623.6 $[M+4H]^{4+}$, 831.0 $[M+3H]^{3+}$, 1246.0 $[M+2H]^{2+}$ and *m/z* = 2489.7 $[M+H]^+$ were observed in ESI-MS and MALDI-TOF spectra, respectively (**see Figs. S5 and S6**).

For the synthesis of the Ru(II)-PNA oligomer, PNA2, the complex C1 was coupled, on the solid-phase, to the constituted PNA sequence, PNA1, using HATU-mediated condensation followed by its cleavage from resin using a TFA/TIS/phenol 85/5/10 (v/v/v) mixture (**Scheme 2**). Extended coupling time (ca. 10 h) was allowed to ensure an excellent yield for the solid-phase attachment of C1 to the PNA sequence. As before, PNA2 was successfully synthesized and isolated as an orange solid. The MALDI-TOF spectrum and LC-MS (refer to **Fig. S7**) provided consolidated and unambiguous proof on the identity of the purified oligomer, PNA2.

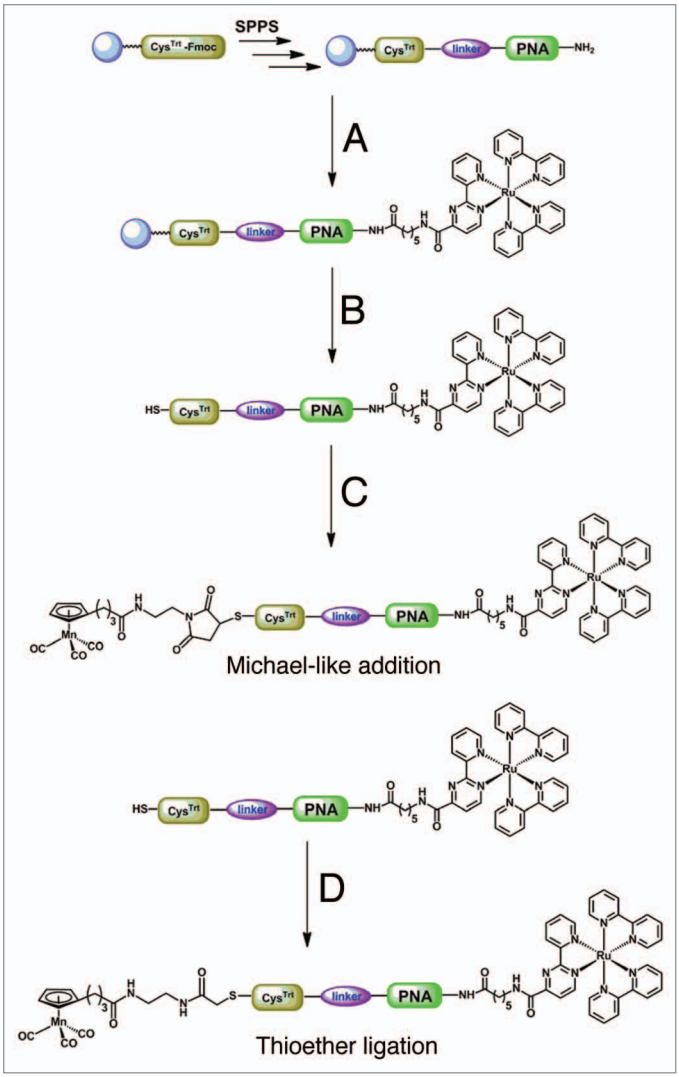


Scheme 1. Synthesis of the cymantrenyl derivatives C2 and C3. Reagents and conditions: (**A**) *N*-(2-aminoethyl)maleimide trifluoroacetate, HATU, NEt_3, DMF, rt, 12 h, 61%; (**B**) *N*-Boc-ethylenediamine, HATU, DMAP, NEt_3, DMF, rt, 12 h, 76%; (**C**) 1. TFA/CH_2Cl_2 (1:1 v/v), rt, 5 h; 2. Chloroacetylchloride, NEt_3, CH_2Cl_2, rt, 16 h, 61%.

Synthesis of ruthenium(II)-cymantrenyl PNA oligomers. With the synthesized PNA sequences PNA1 and PNA2 in hand, we proceeded toward the preparation of their cymantrenyl adducts PNA3 and PNA4 (**Table 1**). The synthesis of succinimide thioether analog PNA3, in solution, was first attempted on the non-metalated sequence, PNA1. The preparation followed Michael-like addition of thiol-functionalized PNA1 to cymantrenyl maleimide derivative C2. The high specificity associated with such thiol-based Michael additions offers an extremely effective and convenient approach to bioconjugation.[52-56] The successful formation of the thiol-maleimide adduct was established from the LC-MS spectrum (**Fig. S8**). Peaks for the $[M+4H+Na]^{5+}$, $[M+3H+Na]^{4+}$ and $[M+2H+Na]^{3+}$ ions were observed at *m/z* = 585.1, 731.1 and 974.3, respectively. The presence of the intact manganese tricarbonyl unit in the formed thiol maleimide adduct, PNA3, was further verified through IR spectroscopy. As anticipated, sharp bands at 1930 and 2017 cm^{-1} for the characteristic C≡O vibrational stretches were observed in the IR spectrum (**Fig. S12**). Previously, we have reported similar approach to confirm the presence of metal-carbonyls using IR spectroscopy in a hetero-di-organometallic PNA oligomer incorporating individual $Re(CO)_3$ and $Mn(CO)_3$ cores.[45] Note that other metal carbonyl compounds have been coupled to biomolecules with a view to their detection using the so-called carbonylmetalloimmunoassay (CMIA).[57,58] With the experimental conditions for the formation of thiol-maleimide based PNA-cymantrenyl adduct (PNA3) being optimized, a similar synthesis was attempted using the Ru(II)-PNA oligomer, PNA2. The formation of the desired Ru(II)-cymantrenyl PNA oligomer, PNA4, was confirmed by peaks at *m/z* = 596.3, 715.3 and 893.8 for $[M+5H+Na]^{6+}$, $[M+4H+Na]^{5+}$and $[M+3H+Na]^{4+}$ ions in LC-MS and metal carbonyl stretches (2008 and 2074 cm^{-1}) in the IR spectrum.

As an alternative approach for PNA hetero-metalation, the scope of thioether ligation strategy was also assessed in this work. As for the Michael-like addition, the ligation reaction was initially tested for the non-metalated PNA sequence, PNA1. Reaction between chloroacetyl derivative, C3, and PNA1 proceeded efficiently under basic conditions, in presence of potassium iodide. The LC-MS trace for the HPLC-purified thioether PNA5 showed peaks for the respective ion fragments (refer to characterization details in the Experimental Section and **Fig. S10**) and weak but well defined bands at 2062 and 1996 cm^{-1} in the IR spectrum, originating from C≡O vibrational stretches of the manganese tricarbonyl entity (**Fig. S13** in SI). To further extend this proof-of-principle study, thioether ligation between C3 and Ru(II)-PNA oligomer, PNA2, was also attempted. The ligated product PNA6 containing the Ru(II)-polypyridyl core at the one end and the manganese tricarbonyl core on the other was successfully isolated after RP-HPLC (**Fig. S11**). As anticipated, the organometallic C3 backbone and the Ru(II)-polypyridyl unit of PNA2 did not impose any unfavorable steric constraints for the formation of the desired di-metalated thioether. Interestingly, the IR spectra of the cymantrenyl PNAs prepared in this work, in general, show the carbonyl vibrational stretches to be shifted in comparison to the parent cymantrenyl unit, C2. The origin of these interesting shifts is unclear at present and will be the subject of future investigation.

This work, to the best of our knowledge, is the first attempt on utilizing thioether ligation for preparation of hetero-metalated PNA oligomers which we believe will be applicable for the



Scheme 2. Synthesis of the ruthenium(II)-cymantrenyl PNA oligomers. Reagents and conditions: (**A**) Complex C1, HATU, DMF, 10 h; (**B**) TFA/TIS/Phenol (85/5/10, v/v/v); (**C**) Complex C2, DIPEA, acetonitrile/water (ca. 1/1, v/v), 30°C, 48 h; (**D**) Complex C3, KI, DIPEA, water/acetonitrile (ca. 1/1, v/v), 30°C, 48 h. SPPS, standard Fmoc solid-phase PNA/peptide synthesis; linker, Fmoc-AEEA-OH. See Experimental Section for details.

coupling of various combinations of the metal complexes and thiol-functionalized metal-containing PNA oligomers.

Experimental Section

Materials. All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or distilled using standard procedures.[59] Deionised water was used for all reactions in aqueous solution. Reagents and solvents for solid-phase peptide synthesis were of HPLC grade and purchased from Acros, Aldrich/Sigma/Fluka, E. Merck and IRIS Biotech and were used without further purification. The preloaded polystyrene resins were purchased from Rapp Polymers. Only L-amino acids were used throughout. PNA monomers were purchased from Link Technologies. Thin layer chromatography (TLC) was performed using silica gel 60 F-254 (Merck) plates with detection of spots being achieved by exposure UV light. Column chromatography was done using Silica gel 60 (0.040–0.063 mm mesh, Merck). Eluent mixtures are expressed as volume to volume (v/v) ratios.

Instrumentation and methods. ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on AV2–401 spectrometer, at room temperature. The chemical shifts, δ, are reported in ppm (parts per million). The signals from the residual protons of deuterated solvent have been used as an internal reference.[60] The abbreviations for the peak multiplicities are as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). ESI mass spectrometry was performed using a Bruker Esquire 6000 spectrometer. In the assignment of the mass spectra, the most intense peak is listed. High-resolution accurate mass spectra were recorded with Bruker maXis QTof high-resolution mass spectrometer (Bruker Daltonics, Bremen, Germany). Infrared spectra were directly recorded on Perkin-Elmer FTIR spectrometer fitted with an ATR platform. In case of cymantrenyl PNAs (PNA3–PNA6), their aqueous solutions were directly used for measuring the IR spectrum. Peak intensities are given as broad (br), very strong (vs), strong (s), medium (m) and weak (w). Microanalysis was performed on a LecoCHNS-932 elemental analyzer. The matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra were measured on a Bruker Daltonics Autoflex. The experiments were performed either in linear or reflector mode with positive polarity using sinapinic acid or α-cyano-4-hydroxy-cinnamic acid as the matrix. HPLC purification of PNAs was performed on a Varian ProStar system equipped with a diode array UV/Vis spectrometer and a LiChroCART® 250–10 RP-18 semi-prep column (5 μm particle size, 100 Å pore size, 10 × 250 mm. Flow rate: 4 $mLmin^{-1}$). Chromatographic separations were performed with a linear gradient of A (distilled water containing 0.1% v/v TFA) and B [acetonitrile (Sigma-Aldrich HPLC grade), containing 0.1% v/v TFA]. Preparative runs: t = 0 min, 5% B; t = 12 min, 15% B; t = 32 min, 40% B; t = 50 min, 80% B; t = 51 min, 100% B; t = 56 min, 100% B; t = 61 min, 5% B. The LC-MS spectrum of PNA sequences was measured on an Acquity™ Waters system equipped with a PDA detector and an auto sampler using an Agilent Zorbax 300SB-C18 analytical column (3.5 mm particle size, 300 Å pore size, 150 × 4.6 mm). The LC was coupled to an Esquire HCT from Bruker for the MS measurements. The LC run (flow rate: 0.3 $mLmin^{-1}$) was performed with a linear gradient of A (distilled water containing 0.1% v/v formic acid) and B [acetonitrile (Sigma-Aldrich HPLC-grade], containing 0.1% v/v formic acid); t = 0 min, 5% B; t = 3 min, 5% B; t = 17 min, 100% B; t = 20 min, 100% B; t = 25 min, 5% B. The purity of all PNA sequences, as analyzed from the corresponding LC-MS traces, was found to be greater than 95%. *Microanalysis* was performed on a LecoCHNS-932 elemental analyzer.

Synthesis. Compounds C1,[61] 4-cymantrenylbutyric acid (1),[62] and *N*-(2-aminoethyl)maleimide trifluoroacetate salt[52] were synthesized according to literature procedures. All the characterization data was in agreement with the literature reports. All reactions with cymantrenyl derivatives were protected from light

by wrapping assemblies with aluminum foil and performed in absence of light, as much as possible.

Compound C2. HATU (0.196 g, 0.52 mmol) and triethylamine (0.069 g, 0.68 mmol) were added to a 4-cymantrenylbutyric acid (1) (0.100 g, 0.34 mmol) in dimethylformamide (10 mL), and the resulting solution was stirred at room temperature for 20 min. Following this, *N*-(2-aminoethyl)maleimide trifluoroacetate (0.114 g, 0.45 mmol) was added and the stirring was continued for 12 h. The reaction mixture was concentrated under reduced pressure and the residue obtained was dissolved in dichloromethane (15 mL) and filtered. The dichloromethane solution was washed with 1 M HCl (2 × 10 mL), water (2 × 10 mL) and brine (1 × 15 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness to obtain the crude product. Purification of the crude product by column chromatography (SiO_2, 50% ethylacetate/hexane) afforded compound C2 as yellow solid. Yield: 0.086 g (61%). R_f = 0.44 in 50% ethylacetate/hexane. Anal. calcd. for $C_{18}H_{18}MnN_2O_6$ (%): C, 52.31; H, 4.39; N, 6.78. Found: C, 52.53; H, 4.15; N, 6.45. IR (ATR): ν 3248m (N-H), 3082m (C-H), 2946w (C-H), 2010vs (C≡O), 1905vs (C≡O), 1703vs (C=O), 1635 m (C=O), 1564m, 1436m, 1408m, 1265w, 1173m, 1103w, 827m, 668m, 631s cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 6.73 (s, 2H, 2 × C*H)* 4.64 (m, 4H, C_5H_4), 3.70 (m, 2H, CH_2), 3.48–3.47 (m, 2H, CH_2), 2.28–2.19 (m, 4H, 2 × CH_2), 1.84–1.81 (m, 2H, -$CH_2CH_2CH_2$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 225.2, 171.2, 134.5, 106.4, 82.4, 82.0, 39.2, 37.9, 35.9, 27.8, 26.8 ppm. MS (ESI^+): *m/z* 435.0 $[M+Na]^+$.

Compound 2. The synthesis of compound 2 was performed via the same method as for the preparation of C2, but using 4-cymantrenylbutyric acid (1) (0.200 g, 0.69 mmol), HATU (0.525 g, 1.38 mmol), triethylamine (0.139 g, 1.38 mmol), DMAP (8.5 mg) and *N*-Boc-ethylenediamine (0.221 g, 1.38 mmol) in dimethylformamide (10 mL). Purification by silica gel column chromatography using 50% ethylacetate/hexane as eluent, yielded the pure product as yellow oil. Yield: 0.226 mg (76%). R_f = 0.25 in 25% ethylacetate/hexane. IR (neat): ν 3317br (N-H), 2975m (C-H), 2936m (C-H), 2016vs (C≡O), 1921vs (C≡O), 1693s (C=O), 1643s (C=O), 1517s, 1251m, 1168m, 756m, 638s cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 4.64 (m, 4H, C_5H_4), 3.37–3.28 (m, 4H, 2 × CH_2), 2.30–2.24 (m, 4H, 2 × CH_2), 1.85 (m, 2H, -$CH_2CH_2CH_2$), 1.45 (s, 9H, ^{t}Bu CH_3) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 225.4, 106.5, 82.3, 82.1, 80.1, 41.3, 40.5, 38.9, 28.6, 28.0, 27.0 ppm. MS (ESI^+): *m/z* 455.1 $[M+Na]^+$. HR-ESI mass spectrum (acetonitrile + NaI): found 455.09834; calcd. for $[C_{19}HMnN_2O_6Na]/z$ 455.09853.

Compound C3. Compound 2 (0.200 g, 0.46 mmol) was deprotected by stirring it for 5 h, at room temperature, in 5 mL trifluoroacetic acid/dichloromethane solution (1:1, v/v). The solvent was evaporated under reduced pressure, and the concentrate was triturated with toluene and ether. The residue was dissolved in dry dichloromethane (10 mL), triethylamine (0.102 g, 1.01 mmol) was added and solution was stirred for 10 min at room temperature. Subsequently, chloroacetylchloride (0.055 g, 0.49 mmol) solution in dichloromethane (5 mL) was slowly added at 0°C, followed by further stirring for 16 h at room temperature. Cold water (10 mL) was added to the reaction solution and extracted with dichloromethane (2 × 20 mL). The combined organic extracts were back washed water and dried over Na_2SO_4. The solution was filtered and concentrated under reduced pressure, and the residue was purified using silica gel column chromatography using 50% ethyl acetate/hexane as eluent to afford compound **C3** as pale white solid. Yield: 0.115 mg (61%). R_f = 0.13 in 50% ethylacetate/hexane. Anal. calcd. for $C_{16}H_{18}ClMnN_2O_5$ (%): C, 47.02; H, 4.44; N, 6.85. Found: C, 47.38; H, 4.37; N, 6.54. IR (ATR): ν 3266s (N-H), 3087m (C-H), 2946m (C-H), 2012vs (C≡O), 1927vs (C≡O), 1905vs (C≡O), 1661s (C=O), 1635s (C=O), 1555s, 1241m, 954w, 838w, 668m, 635s cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 7.17 (br s, 1H, -CON*H*), 6.08 (br s, 1H, -CON*H*) 4.64 (m, 4H, C_5H_4), 4.04 (s, 2H, -$COCH_2$Cl), 3.46 (m, 4H, 2 × CH_2), 2.29–2.27 (m, 4H, 2 × CH_2), 1.85 (m, 2H, -$CH_2CH_2CH_2$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 225.3, 106.2, 82.2, 82.0, 42.6, 40.7, 39.9, 35.9, 27.8, 26.8 ppm. MS (ESI^+): *m/z* 408.5 $[M+H]^+$.

Table 1. PNA sequences prepared in this study

PNA code	Sequence[a]
PNA1	H-gly-a-a-g-t-c-t-g-c-linker-cys-NH_2
PNA2	*C1*-a-a-g-t-c-t-g-c-linker-cys-NH_2
PNA3	H-gly-a-a-g-t-c-t-g-c-linker-CH($CONH_2$)CH_2-S-*C2*
PNA4	*C1*-a-a-g-t-c-t-g-c-linker-CH($CONH_2$)CH_2-S-*C2*
PNA5	H-gly-a-a-g-t-c-t-g-c-linker-CH($CONH_2$)CH_2-S-*C3*
PNA6	*C1*-a-a-g-t-c-t-g-c-linker-CH($CONH_2$)CH_2-S-*C3*

[a]Small letters a, t, g, c denote PNA monomers while the amino acids are represented using standard three letter codes. PNA sequences are written from *N*- to *C*- terminus. Linker used: Fmoc-AEEA-OH.

Synthesis of PNA oligomers. The SPPS of PNA1 was manually performed in one-way polypropylene syringes (5 mL), equipped with a frit, using polystyrene resin beads of TentaGel S RAM Cys(Trt)Fmoc (36 mg, 0.21 mmol/g), as previously reported.[40,47,49,50]

Synthesis of Ru-PNA oligomer (PNA2). *N*-terminus solid-phase insertion of $[Ru(bpy)_2(Cpp\text{-}NH\text{-}Hex\text{-}COOH)](PF_6)_2$ (C1) on PNA1 was achieved by following the general SPPS procedure. Coupling followed the Fmoc deprotection of the last PNA monomer of the oligomeric sequence. A solution of C1 (5 eq.) in DMF, preactivated in eppendorf tube for 2 min with HATU (4.5 eq.), DIPEA and 2,6-lutidine (10 eq. each), was added to PNA1 preloaded polystyrene resin. A longer reaction time of 10 h was allowed in order to ensure a full coupling.

Cleavage of the PNA from the resin. Before cleavage, the resin containing the PNA was contracted with methanol and dried. The non-metallic PNA (PNA1) was cleaved using a mixture of trifluoroacetic acid/water/triisopropylsilane 95/2.5/2.5 (v/v/v) while the metal-containing PNA (PNA2) was cleaved using a mixture of trifluoroacetic acid/triisopropylsilane/phenol 85/5/10 (v/v/v) [3 × 400 μL (1.5 h each)]. Trifluoroacetic acid was removed from the resulting solutions under high vacuum before the crude oligomers being precipitated with ice-cold ether. The solids were centrifuged, washed with ice-cold ether and finally air-dried. The crude oligomers were purified with RP-HPLC, and characterized by ESI-MS and MALDI-TOF mass spectrometry.

Solution phase synthesis of cymantrenyl-PNA adducts (PNA3-PNA6). Stock solutions of PNA1 and PNA2 in nanopure water were used for the reaction. The strand concentration of PNA oligomer was estimated by UV spectroscopy at 80°C using the sum of molar extinction coefficients at 260 nm (ε_{260}) for PNA nucleobases present in the oligomeric strands ($\varepsilon_{PNA,A}$ = 13700 $M^{-1}cm^{-1}$, $\varepsilon_{PNA,G}$ = 11700 $M^{-1}cm^{-1}$, $\varepsilon_{PNA,C}$ = 6600 $M^{-1}cm^{-1}$, $\varepsilon_{PNA,T}$ = 8600 $M^{-1}cm^{-1}$). The molar extinction coefficient of $[Ru(bpy)_2(Cpp\text{-}NH\text{-}Hex\text{-}COOH)](PF_6)_2$ (C1), at 260 nm, ε_{260} = 13300 $M^{-1}cm^{-1}$ was estimated using $[Ru(bpy)_3]^{2+}$ as model compound and determined from the slope of the absorption (A_{260}) vs. concentration curve.[47,61]

Synthesis of cymantrenyl maleimide-PNA adducts (PNA3 and PNA4). A mixture of PNA1 (1.6 μmol) or PNA2 (0.8 μmol), compound C2 (1.1 eq) and 0.5 μL of diisopropylethylamine (DIPEA) in 150 μL acetonitrile was shaken for 48 h on mechanical shaker (930 rpm, 30°C). The reaction solution was freeze-dried and crude product was purified by RP-HPLC. The PNA oligomers were characterized by ESI-MS and IR spectroscopy.

Synthesis of thioether ligated cymantrenyl-PNA adducts (PNA5 and PNA6). PNA1 (0.8 μmol) or PNA2 (0.5 μmol), compound C3 (1.0 eq), KI (20 eq) and diisopropylethylamine (DIPEA) (60 eq) were successively added in 150 μL acetonitrile. The reaction mixture was sonicated for 10 min and thereafter shaken on mechanical shaker (930 rpm, 30°C) for 48 h. Following this, the reaction solution was freeze-dried and crude product was purified by RP-HPLC. The thioether ligated PNAs were characterized by ESI-MS and IR spectroscopy.

Characterization of PNA1. ESI-MS: *m/z* 499.1 $[M+5H]^{5+}$, 623.6 $[M+4H]^{4+}$, 831.0 $[M+3H]^{3+}$, 1246.0 $[M+2H]^{2+}$. MALDI-TOF: *m/z* 2489.7 $[M+H]^{+}$.

Characterization of PNA2. ESI-MS: *m/z* 524.6 $[M+6H]^{6+}$, 629.3 $[M+5H]^{5+}$, 786.3 $[M+4H]^{4+}$, 1048.2 $[M+3H]^{3+}$. MALDI-TOF: *m/z* 3143.2 $[M+H]^{+}$.

Characterization of PNA3. HPLC: t_R = 23.4 min. ESI-MS: *m/z* 585.1 $[M+4H+Na]^{5+}$, 731.1 $[M+3H+Na]^{4+}$, 974.3 $[M+2H+Na]^{3+}$. IR (ATR): ν 2017s (C≡O), 1930s (C≡O) cm^{-1}.

Characterization of PNA4. HPLC: t_R = 24.2 min. ESI-MS: *m/z* 596.3 $[M+5H+Na]^{6+}$, 715.3 $[M+4H+Na]^{5+}$, 893.8 $[M+3H+Na]^{4+}$. IR (ATR): ν 2074w (C≡O), 2008w (C≡O) cm^{-1}.

Characterization of PNA5. HPLC: t_R = 25.6 min. ESI-MS: m/z 573.5 $[M+5H]^{5+}$, 716.5 $[M+4H]^{4+}$, 955.0 $[M+3H]^{3+}$. IR (ATR): ν 2062m (C≡O), 1996m (C≡O) cm^{-1}.

Characterization of PNA6. HPLC: t_R = 26.8 min. ESI-MS: *m/z* 586.6 $[M+6H]^{6+}$, 703.6 $[M+5H]^{5+}$, 879.3 $[M+4H]^{4+}$. IR (ATR): ν 2043w (C≡O), 1982w (C≡O) cm^{-1}.

Conclusion

In this study, we have demonstrated the feasibility of preparing covalently heterometalated PNA cores by taking the synthesis of Ru(II)-cymantrenyl PNA oligomers PNA4 and PNA6 as an example. The application of solution based Michael-like addition and thioether ligation reaction, in combination with conventional solid-phase peptide coupling, has been showcased as an easy and efficient method for constructing multimetalic PNA oligomers from thiol-functionalised PNA oligomers and appropriately designed metal-containing building blocks like C1–C3. While thiol functionalisation of PNAs was conveniently achieved from the cysteine preloaded resin, taking advantage of the affinity of thiols for maleimide as well as for haloacetyl moieties, highly specific metalation could be achieved through Michael-like addition and thioether ligation reactions. Both approaches hold tremendously strong potential for future application in preparation of diverse library of multi-heterometallic PNA constructs.

Acknowledgments

This work was supported by the Swiss National Science Foundation (SNSF Professorship to G.G.), the University of Zurich (G.G.) and the Australian Research Council through the Australian Centre of Excellence for Electromaterials Science (L.S.). The authors thank Ms. Anna Leonidova for her kind help with the MALDI-TOF measurements.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/artificialdna/article/24019

References

1. Nielsen PE, Egholm M, Berg RH, Buchardt O. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 1991; 254:1497-500; PMID:1962210; http://dx.doi.org/10.1126/science.1962210.
2. Nielsen PE. Targeted gene repair facilitated by peptide nucleic acids (PNA). [and references therein]. Chembiochem 2010; 11:2073-6; PMID:20872391; http://dx.doi.org/10.1002/cbic.201000346.
3. Nielsen PE. Peptide nucleic acids (PNA) in chemical biology and drug discovery. Chem Biodivers 2010; 7:786-804; PMID:20397216; http://dx.doi.org/10.1002/cbdv.201000005.
4. Nielsen PE. The many faces of PNA. Lett Pept Sci 2004; 10:135-47; http://dx.doi.org/10.1007/s10989-004-4860-7.
5. Wang J. PNA biosensor for nucleic acid detection. In: Nielsen PE, Egholm M, eds. Peptide Nucleic Acids, Protocols and Applications. Wymondham, Uk: Horizon Scientific Press, 1999:155-61, and references therein.
6. Nielsen PE, Egholm M, eds. Peptide Nucleic Acids, Protocols and Applications. Wymondham, UK: Horizon Scientific Press, 1999.
7. Beck R. Solid Phase Syntesis of PNA Oligomers. In: Nielsen PE, ed. Peptide Nucleic Acids: Methods and Protocols (Methods in Molecular Biology). Totowa, NJ: Humana Press Inc., 2002:29-41.
8. Ferri E, Donghi D, Panigati M, Prencipe G, D'Alfonso L, Zanoni I, et al. Luminescent conjugates between dinuclear rhenium(I) complexes and peptide nucleic acids (PNA) for cell imaging and DNA targeting. Chem Commun (Camb) 2010; 46:6255-7; PMID:20668778; http://dx.doi.org/10.1039/c0cc00450b.
9. Baldoli C, Cerea P, Giannini C, Licandro E, Rigamonti C, Maiorana S. The Metal-Conjugated Peptide Nucleic Acid Challenge. Synlett 2005; 2005:1984-94.
10. Wilhelmsson LM, Holmén A, Lincoln P, Nielsen PE, Nordén B. A highly fluorescent DNA base analogue that forms Watson-Crick base pairs with guanine. J Am Chem Soc 2001; 123:2434-5; PMID:11456897; http://dx.doi.org/10.1021/ja0025797.
11. Gasser G, Sosniak AM, Metzler-Nolte N. Metal-containing peptide nucleic acid conjugates. [and references therein]. Dalton Trans 2011; 40:7061-76; PMID:21541385; http://dx.doi.org/10.1039/c0dt01706j.
12. Dose C, Seitz O. Single nucleotide specific detection of DNA by native chemical ligation of fluorescence labeled PNA-probes. Bioorg Med Chem 2008; 16:65-77; PMID:17499998; http://dx.doi.org/10.1016/j.bmc.2007.04.059.
13. Metzler-Nolte N. Conjugates of Peptides and PNA with Organometallic Complexes: Syntheses and Applications. In: Jaouen G, ed. Bioorganometallics: Biomolecules, Labeling, Medicine. Weinheim: Wiley-VCH, 2006:125-79.

14. Krämer R, Mokhir A. Metal complex derivatives of peptide nucleic acids (PNA). Met Ions Life Sci 2012; 10:319-40; PMID:22210345; http://dx.doi.org/10.1007/978-94-007-2172-2_12.
15. Wierzbinski E, de Leon A, Davis KL, Bezer S, Wolak MA, Kofke MJ, et al. Charge transfer through modified peptide nucleic acids. Langmuir 2012; 28:1971-81; PMID:22217076; http://dx.doi.org/10.1021/la204445u.
16. de Leon AR, Olatunde AO, Morrow JR, Achim C. Binding of Eu(III) to 1,2-hydroxypyridinone-modified peptide nucleic acids. Inorg Chem 2012; 51:12597-9; PMID:23148561; http://dx.doi.org/10.1021/ic301790v.
17. Ma Z, Olechnowicz F, Skorik YA, Achim C. Metal binding to ligand-containing peptide nucleic acids. Inorg Chem 2011; 50:6083-92; PMID:21634382; http://dx.doi.org/10.1021/ic200138b.
18. Franzini RM, Watson RM, Patra GK, Breece RM, Tierney DL, Hendrich MP, et al. Metal binding to bipyridine-modified PNA. Inorg Chem 2006; 45:9798-811; PMID:17112277; http://dx.doi.org/10.1021/ic0609610.
19. Mokhir A, Krämer R, Wolf H. Zn2+-dependent peptide nucleic acids probes. J Am Chem Soc 2004; 126:6208-9; PMID:15149199; http://dx.doi.org/10.1021/ja0397613.
20. Xavier C, Giannini C, Dall'Angelo S, Gano L, Maiorana S, Alberto R, et al. Synthesis, characterization, and evaluation of a novel $^{99m}Tc(CO)_3$ pyrazolyl conjugate of a peptide nucleic acid sequence. J Biol Inorg Chem 2008; 13:1335-44; PMID:18777182; http://dx.doi.org/10.1007/s00775-008-0419-y.
21. Whitney A, Gavory G, Balasubramanian S. Site-specific cleavage of human telomerase RNA using PNA-neocuproine.Zn(II) derivatives. Chem Commun (Camb) 2003; 36-7; PMID:12610953; http://dx.doi.org/10.1039/b210135a.
22. Watson RM, Skorik YA, Patra GK, Achim C. Influence of metal coordination on the mismatch tolerance of ligand-modified PNA duplexes. J Am Chem Soc 2005; 127:14628-39; PMID:16231915; http://dx.doi.org/10.1021/ja051336h.
23. Mardirossian G, Lei K, Rusckowski M, Chang F, Qu T, Egholm M, et al. In vivo hybridization of technetium-99m-labeled peptide nucleic acid (PNA). J Nucl Med 1997; 38:907-13; PMID:9189140.
24. Lewis MR, Jia F, Gallazzi F, Wang Y, Zhang J, Shenoy N, et al. Radiometal-labeled peptide-PNA conjugates for targeting bcl-2 expression: preparation, characterization, and in vitro mRNA binding. Bioconjug Chem 2002; 13:1176-80; PMID:12440850; http://dx.doi.org/10.1021/bc025591s.
25. Miyajima Y, Ishizuka T, Yamamoto Y, Sumaoka J, Komiyama M. Origin of high fidelity in target-sequence recognition by PNA-Ce(IV)/EDTA combinations as site-selective DNA cutters. J Am Chem Soc 2009; 131:2657-62; PMID:19199631; http://dx.doi.org/10.1021/ja808290e.
26. Zelder FH, Mokhir AA, Krämer R. Sequence selective hydrolysis of linear DNA using conjugates of Zr(IV) complexes and peptide nucleic acids. Inorg Chem 2003; 42:8618-20; PMID:14686836; http://dx.doi.org/10.1021/ic034896v.
27. Belousoff MJ, Gasser G, Graham B, Tor Y, Spiccia L. Binding of HIV-1 TAR mRNA to a peptide nucleic acid oligomer and its conjugates with metal-ion-binding multidentate ligands. J Biol Inorg Chem 2009; 14:287-300; PMID:19015900; http://dx.doi.org/10.1007/s00775-008-0448-6.
28. Hudson RHE, Li G, Tse J. The use of Sonogashira coupling for the synthesis of modified uracil peptide nucleic acid. Tetrahedron Lett 2002; 43:1381-6; http://dx.doi.org/10.1016/S0040-4039(02)00024-2.
29. Baldoli C, Falciola L, Licandro E, Maiorana S, Mussini P, Ramani P, et al. A new Ferrocene Conjugate of a Tyrosine PNA Monomer: Synthesis and Electrochemical Properties. J Organomet Chem 2004; 689:4791-802; http://dx.doi.org/10.1016/j.jorganchem.2004.09.049.
30. Baldoli C, Giannini C, Licandro E, Maiorana S, Zinzalla G. A Thymine-PNA Monomer as New Isocyanide Component in the Ugi Reaction: A Direct Entry to PNA Dimers. Synlett 2004; 6:1044-8; http://dx.doi.org/10.1055/s-2004-822886.
31. Baldoli C, Rigamonti C, Maiorana S, Licandro E, Falciola L, Mussini PR. A new triferrocenyl-tris(hydroxymethyl)aminomethane derivative as a highly sensitive electrochemical marker of biomolecules: application to the labelling of PNA monomers and their electrochemical characterization. Chemistry 2006; 12:4091-100; PMID:16544344; http://dx.doi.org/10.1002/chem.200501466.
32. Baldoli C, Licandro E, Maiorana S, Resemini D, Rigamonti C, Falciola L, et al. Electrochemical Activity of New Ferrocene-Labelled PNA monomers to be applied for DNA detection: Effects of the molecular Structure and of the Solvent. J Electroanal Chem 2005; 585:197-205; http://dx.doi.org/10.1016/j.jelechem.2005.08.010.
33. Gasser G, Belousoff MJ, Bond AM, Spiccia L. Facile synthesis and detailed characterization of a new ferrocenyl uracil peptide nucleic acid monomer. J Org Chem 2006; 71:7565-73; PMID:16995660; http://dx.doi.org/10.1021/jo060868t.
34. Gasser G, Spiccia L. Synthesis of a ferrocenyl uracil PNA monomer for insertion into PNA sequences. J Organomet Chem 2008; 693:2478-82; http://dx.doi.org/10.1016/j.jorganchem.2008.05.009.
35. Verheijen JC, van Der Marel GA, van Boom JH, Metzler-Nolte N. Transition metal derivatives of peptide nucleic acid (PNA) oligomers-synthesis, characterization, and DNA binding. Bioconjug Chem 2000; 11:741-3; PMID:11087319; http://dx.doi.org/10.1021/bc0000740.
36. Hamzavi R, Happ T, Weitershaus K, Metzler-Nolte N. The use of 3,3-bis(2-imidazolyl)propionic acid (bip-OH) as a new chelating ligand for Re(CO)3 and Ru complexes: Formation of organometallic PNA oligomers with (bip)Re(CO)3 and their interaction with complementary DNA. J Organomet Chem 2004; 689:4745-50; http://dx.doi.org/10.1016/j.jorganchem.2004.09.009.
37. Hüsken N, Gebala M, La Mantia F, Schuhmann W, Metzler-Nolte N. Mechanistic studies of Fc-PNA(·DNA) surface dynamics based on the kinetics of electron-transfer processes. Chemistry 2011; 17:9678-90; PMID:21735492; http://dx.doi.org/10.1002/chem.201003764.
38. Maurer A, Kraatz HB, Metzler-Nolte N. Synthesis and Electrochemical Characterization of Metallocene-PNA Oligomers. Eur J Inorg Chem 2005; 3207-10; http://dx.doi.org/10.1002/ejic.200500316.
39. Mari C, Panigati M, D'Alfonso L, Zanoni I, Donghi D, Sironi L, et al. Luminescent Conjugates between Dinuclear Rhenium Complexes and Peptide Nucleic Acids (PNA): Synthesis, Photophysical Characterization, and Cell Uptake. Organometallics 2012; 31:5918-28; http://dx.doi.org/10.1021/om3004515.
40. Hüsken N, Gasser G, Köster SD, Metzler-Nolte N. "Four-potential" ferrocene labeling of PNA oligomers via click chemistry. Bioconjug Chem 2009; 20:1578-86; PMID:19586015; http://dx.doi.org/10.1021/bc9001272.
41. Gasser G, Hüsken N, Köster SD, Metzler-Nolte N. Synthesis of organometallic PNA oligomers by click chemistry. Chem Comm 2008:3675-7.
42. Köster SD, Dittrich J, Gasser G, Hüsken N, Hernandez I, Jios JL, et al. Spectroscopic and Electrochemical Studies of Ferrocenyl Triazole Amino Acid and Peptide Bioconjugates synthesized by Click Chemistry. Organometallics 2008; 27:6326-32; http://dx.doi.org/10.1021/om800639m.
43. Sosniak A, Gasser G, Metzler-Nolte N. Thermal Melting Studies of Modified PNA Oligomers. Org Biomol Chem 2009; 7:4992-5000; PMID:19907791; http://dx.doi.org/10.1039/b913964h.
44. Patra M, Gasser G, Bobukhov D, Merz K, Shtemenko AV, Metzler-Nolte N. Sequential insertion of three different organometallics into a versatile building block containing a PNA backbone. Dalton Trans 2010; 39:5617-9; PMID:20485811; http://dx.doi.org/10.1039/c003598j.
45. Gasser G, Neumann S, Ott I, Seitz M, Heumann R, Metzler-Nolte N. Preparation and Biological Evaluation of Di-Hetero Organometallic-Containing PNA Bioconjugates. Eur J Inorg Chem 2011; 5471-8; http://dx.doi.org/10.1002/ejic.201100734.
46. Joshi T, Gasser G, Martin LL, Spiccia L. Specific Uptake and Interactions of Peptide Nucleic Acid Derivatives with Biomimetic Membranes. RSC Adv 2012; 2:4703-12; http://dx.doi.org/10.1039/c2ra20462b.
47. Joshi T, Barbante GJ, Francis PS, Hogan CF, Bond AM, Gasser G, et al. Electrochemiluminescent monomers for solid support syntheses of Ru(II)-PNA bioconjugates: multimodal biosensing tools with enhanced duplex stability. Inorg Chem 2012; 51:3302-15; PMID:22339152; http://dx.doi.org/10.1021/ic202761w.
48. Joshi T, Barbante GJ, Francis PS, Hogan CF, Bond AM, Spiccia L. Electrochemiluminescent peptide nucleic acid-like monomers containing Ru(II)-dipyridoquinoxaline and Ru(II)-dipyridophenazine complexes. Inorg Chem 2011; 50:12172-83; PMID:22040143; http://dx.doi.org/10.1021/ic201911f.
49. Gasser G, Pinto A, Neumann S, Sosniak AM, Seitz M, Merz K, et al. Synthesis, characterisation and bioimaging of a fluorescent rhenium-containing PNA bioconjugate. Dalton Trans 2012; 41:2304-13; PMID:22183093; http://dx.doi.org/10.1039/c2dt12114j.
50. Gasser G, Jäger K, Zenker M, Bergmann R, Steinbach J, Stephan H, et al. Preparation, 99mTc-labeling and biodistribution studies of a PNA oligomer containing a new ligand derivative of 2,2'-dipicolylamine. J Inorg Biochem 2010; 104:1133-40; PMID:20674031; http://dx.doi.org/10.1016/j.jinorgbio.2010.06.011.
51. Gasser G, Sosniak AM, Leonidova A, Braband H, Metzler-Nolte N. Towards the Preparation of novel Re/99mTc Tricarbonyl-Containing Peptide Nucleic Acid Bioconjugates. Aust J Chem 2011; 64:265-72; http://dx.doi.org/10.1071/CH11010.
52. Liu F, Soh Yan Ni A, Lim Y, Mohanram H, Bhattacharjya S, Xing B. Lipopolysaccharide neutralizing peptide-porphyrin conjugates for effective photoinactivation and intracellular imaging of gram-negative bacteria strains. Bioconjug Chem 2012; 23:1639-47; PMID:22769015; http://dx.doi.org/10.1021/bc300203d.
53. Baldwin AD, Kiick KL. Tunable degradation of maleimide-thiol adducts in reducing environments. Bioconjug Chem 2011; 22:1946-53; PMID:21863904; http://dx.doi.org/10.1021/bc200148v.
54. Foley TL, Yasgar A, Garcia CJ, Jadhav A, Simeonov A, Burkart MD. Preparation of FRET reporters to support chemical probe development. Org Biomol Chem 2010; 8:4601-6; PMID:20725690; http://dx.doi.org/10.1039/c0ob00322k.
55. Pounder RJ, Stanford MJ, Brooks P, Richards SP, Dove AP. Metal free thiol-maleimide 'Click' reaction as a mild functionalisation strategy for degradable polymers. Chem Commun (Camb) 2008; 5158-60; PMID:18956054; http://dx.doi.org/10.1039/b809167f.

56. Juárez-Hernández RE, Miller PA, Miller MJ. Syntheses of Siderophore-Drug Conjugates Using a Convergent Thiol-Maleimide System. ACS Med Chem Lett 2012; 3:799-803; PMID:23264853; http://dx.doi.org/10.1021/ml300150y.
57. Salmain M, Vessières A, Brossier P, Butler IS, Jaouen G. Carbonylmetalloimmunoassay (CMIA) a new type of non-radioisotopic immunoassay. Principles and application to phenobarbital assay. J Immunol Methods 1992; 148:65-75; PMID:1564336; http://dx.doi.org/10.1016/0022-1759(92)90159-Q.
58. Vessières A, Salmain M, Brossier P, Jaouen G. Carbonyl metallo immuno assay: a new application for Fourier transform infrared spectroscopy. J Pharm Biomed Anal 1999; 21:625-33; PMID:10701430; http://dx.doi.org/10.1016/S0731-7085(99)00169-7.
59. Armarego WLF, Perrin DD. Purification of Laboratory Chemicals. Oxford (UK): Butterworth-Heinemann, 1996.
60. Fulmer GR, Miller AJM, Sherden NH, Gottlieb HE, Nudelman A, Stoltz BM, et al. NMR Chemical Shifts of Trace Impurities: Common Laboratory Solvents, Organics, and Gases in Deuterated Solvents Relevant to the Organometallic Chemist. Organometallics 2010; 29:2176-9; http://dx.doi.org/10.1021/om100106e.
61. Pierroz V, Joshi T, Leonidova A, Mari C, Schur J, Ott I, et al. Molecular and cellular characterization of the biological effects of ruthenium(II) complexes incorporating 2-pyridyl-2-pyrimidine-4-carboxylic acid. J Am Chem Soc 2012; 134:20376-87; PMID:23181418; http://dx.doi.org/10.1021/ja307288s.
62. Patra M, Gasser G, Wenzel M, Merz K, Bandow JE, Metzler-Nolte N. Sandwich and Half-Sandwich Derivatives of Platensimycin: Synthesis and Biological Evaluation. Organometallics 2012; 31:5760-71; http://dx.doi.org/10.1021/om201146c.