Polyhedron 30 (2011) 2387–2390

Contents lists available at ScienceDirect

Polyhedron

journal homepage: www.elsevier.com/locate/poly

Towards peptide-substituted titanocene anticancer drugs

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ARTICLE INFO

Article history:
Received 25 February 2011
Accepted 31 May 2011
Available online 22 June 2011

Keywords:
Bioorganometallic chemistry
Metal-based anticancer drugs
Titanocene dichloride
[3+2] Cycloaddition
Targeting vector
Enkephalin

ABSTRACT

An alkyne-substituted fulvene was transformed via hydridolithiation followed by transmetallation with titanium tetrachloride into *bis*-[*p*-(prop-2-ynyloxy)-benzyl-cyclopentadienyl] titanium(IV) dichloride. Single crystals of this titanocene derivative could be obtained and the structure determined by X-ray diffraction. It showed that this compound crystallises in the space group C2/c with four molecules in the monoclinic cell. The alkyne-substituted titanocene dichloride derivative was then subject to a copper-catalysed azide–alkyne cycloaddition with its azide-functionalised methylester-protected phenylalanine reaction partner in order to form a linking triazole. This reaction was performed under anhydrous conditions employing a dichloromethane/acetonitrile solvent mixture with copper(I) iodide and 2,6-lutidine as the catalyst system. Under these conditions the adduct between the protein mimic and the titanocene was formed without hydrolysing the titanium dichloride moiety.

1. Introduction

In the last years significant progress was made with respect to the development of titanocene anticancer reagents focusing on difficult to treat advanced renal-cell cancer [1,2]. The lead compound Titanocene Y (*bis*-[(*p*-methoxybenzyl)cyclopentadienyl] titanium(IV) dichloride), which was synthesised through hydridolithiation of *p*-anisyl fulvene with Super Hydride ($LiBEt_3H$) followed by transmetallation with $TiCl_4$ [3], exhibits a unique interaction with its target DNA [4] and is not cross-resistant with respect to platinum-based anticancer drugs [5]. Titanocene Y shows good results *in vitro* with respect to cytotoxicity [6] and anti-angiogenesis [7] as well as impressive tumour volume growth reductions *in vivo* [8,9]. Nevertheless, it would be highly attractive to reduce the general toxicity of Titanocene Y by making it more selective with respect to uptake into cancer cells. Therefore, the introduction of a "targeting substituent", which is covalently bonded to the titanocene dichloride moiety is a possible approach to achieve this goal; the tumour-targeting oligopeptide enkephalin would be an obvious choice [10].

The aim of the research is to develop a route to alkyne-substituted titanocene derivatives, which could be easily reacted with azide-substituted biological molecules, such as proteins using Huisgen's well known [3+2] cycloaddition reaction [11], which was rediscovered as the copper catalysed azide–alkyne cycloaddition (CuAAC) or "click reaction" [12]. In this specific experiment, the coupling of a alkyne-substituted titanocene is performed with a low-molecular weight azide-functionalised protein mimic.

2. Experimental

2.1. General conditions

Titanium tetrachloride, Super Hydride ($LiBEt_3H$, 1.0 M solution in THF) and all organic starting materials were obtained from Aldrich Chemical Company and used without further purification. All solvents were dried using standard methods, distilled and collected under an atmosphere of nitrogen prior to use. Manipulations of air and moisture sensitive compounds were done using standard Schlenk techniques, under a nitrogen atmosphere or in a Braun glovebox. NMR spectra were measured on either a Varian 300, 400 or 500 MHz or a Bruker DPX250 spectrometer. Chemical shifts are reported in ppm and are referenced to TMS. Coupling constants (*J*) are quoted in Hertz. $^{13}C\{^1H\}$ assignments were obtained from standard attached proton test (APT) experiments. IR spectra were recorded on a Varian 3100 FT-IR Spectrometer employing KBr or NaCl disks. UV–Vis spectra were recorded on a Varian Cary 50 UV4 Spectrometer. Electrospray ionisation mass spectra (ESI-MS) were recorded on a Bruker Esquire 6000 spectrometer. In the Dublin laboratory CHN analysis was done with an Exeter Analytical CE-440 Elemental Analyser, while Cl was determined in mercurimetric titrations. At the Ruhr-University Bochum elemental analyses were carried out at the RUBiospek department on a Elementar

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doi:10.1016/j.poly.2011.05.036

Hanau vario EL. X-ray diffraction data for the compounds were collected using a Bruker SMART APEX CCD area detector diffractometer. A full sphere of reciprocal space was scanned by phi–omega scans. Pseudo-empirical absorption correction based on redundant reflections was performed by the program SADABS [13]. The structures were solved by direct methods using SHELXS-97 [14] and refined by full matrix least-squares on F^2 for all data using SHELXL-97 [14]. All hydrogen atoms were located in the difference fourier map and allowed to refine freely for titanocene **2**. The data collection details as well as reliability factors are listed in Table 1. Suitable crystals were grown in saturated dichloromethane solution with slow infusion of pentane.

Table 1
Crystallographic refinement data for titanocene **2**.

Identification code	**2**
Empirical formula	$C_{30}H_{26}Cl_2O_2Ti$
Formula weight	537.31
T (K)	100(2)
Wavelength (Å)	0.71073
Crystal system	Monoclinic
Space group	C2/c (#15)
Unit cell dimensions	
a (Å)	25.1349(14)
b (Å)	6.5066(4)
c (Å)	18.7714(11)
α (°)	90
β (°)	124.060(1)
γ (°)	90
V (Å^3)	2543.3(3)
Z	4
D_{calc} (mg/m^3)	1.403
Absorption coefficient (mm^{-1})	0.573
F(0 0 0)	1112
Crystal size (mm)	0.50 × 0.40 × 0.15
θ range for data collection (°)	1.96–32.05
Index ranges	$-37 \leqslant h \leqslant 36$, $-9 \leqslant k \leqslant 9$, $-27 \leqslant l \leqslant 27$
Reflections collected	29,656
Independent reflections	4243 [R_{int} = 0.0205]
Completeness to θ_{max}	95.7%
Absorption correction	Semi-empirical from equivalents
Maximum and minimum transmission	0.9190 and 0.7687
Refinement method	Full-matrix least-squares on F^2
Data/restraints/parameters	4243/0/211
Goodness-of-fit (GOF) on F^2	1.044
Final R indices [$I > 2\sigma(I)$]	R_1 = 0.0338, wR_2 = 0.0893
R indices (all data)	R_1 = 0.0360, wR_2 = 0.0912
Largest difference in peak and hole (e Å^{-3})	0.511 and −0.262

2.2. Synthesis

2.2.1. 6-[p-(Prop-2-ynyloxy)-phenyl] fulvene (**1**)

The synthesis of *p*-(prop-2-ynyloxy) benzaldehyde followed the procedure outlined by Frixa and coworkers [15]. To a Schlenk flask with *p*-(prop-2-ynyloxy) benzaldehyde (2.48 g, 15 mmol) dissolved in 40 mL MeOH, 2.1 mL (31 mmol) freshly cracked cyclopentadiene and 1.9 mL (23 mmol) pyrrolidine were added at room temperature under a nitrogen atmosphere. The solution was stirred at ambient temperature for 20 h to give a dark orange solution. The reaction was quenched with 2.7 mL (46 mmol) acetic acid and 40 mL of water. The product was extracted into $CHCl_3$ (3 × 20 mL), dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure to give a dark red oil. This oil was purified using column chromatography (silica gel, dichloromethane) to give a dark red solid (Yield: 2.12 g, 10.0 mmol, 66%).

^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.46 (t, 1H, OCH_2CCH), 4.65 (d, 2H, OCH_2CCH), 6.69 (m, 2H, C_5H_4), 6.49 (d, 1H, C_5H_4), 6.32 (d, 1H, C_5H_4), 7.02 (d, 2H, J = 7.0, part of C_6H_4), 7.58 (d, 2H, J = 7.5, part of C_6H_4) ppm. ^{13}C NMR ($CDCl_3$, 126 MHz, proton decoupled): 157.4 (C_q–O of C_6H_4), 142.7 (C1 of fulvene), 136.9 (C3, C4 of fulvene), 134.1 (C2, C5 of fulvene), 131.3 (C_q–CH=C), 129.4 (C_q–CH of C_6H_4), 129.0 (C3, C5 of C_6H_4), 114.6 (C2, C6 of C_6H_4), 74.9 (OCH_2CCH)), 77.1 (OCH_2CCH)) 54.8 (OCH_2CCH) ppm. IR absorptions (solid, KBr): 3280, 2956, 2923, 1852, 1756, 1720, 1693, 1671, 1648, 1594, 1469, 1558, 1539, 1507, 1465, 1457, 1438, 1418, 1383, 1369, 1344, 1300, 1262, 1224, 1177, 1152 1139, 1089, 1026, 970, 928, 915, 903, 876, 850, 825, 808, 759, 730, 707, 677, 668, 658, 634, 620, 605, 553, 526 cm^{-1}. UV–Vis (CH_2Cl_2, nm): 228 (ε 8122), 261 (ε 9510), 330 (ε 1735), λ_{max} 394 (ε 451). ESI-MS (pos. mode): m/z 209.37 ([M+H]$^+$) (exact mass for $C_{15}H_{12}O$ = 208.09). *Anal.* Calc. for $C_{15}H_{12}O$: C, 86.5; H, 5.8. Found: C, 85.6; H, 5.9%.

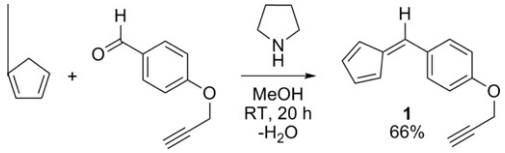
2.2.2. Bis-[p-(prop-2-ynyloxy)-benzyl-cyclopentadienyl] titanium dichloride (**2**)

10.0 mL (10.0 mmol) of 1 M solution of Super Hydride ($LiBEt_3H$) in THF was concentrated by removal of the solvent by heating it to 60 °C under reduced pressure of 10^{-2} mbar for 40 min and then to 90 °C for 20 min in a Schlenk flask. The concentrated Super Hydride was dissolved in 30 mL of dry diethyl ether to give a cloudy white suspension. 1.91 g (9.14 mmol) of the dark red solid 6-(*p*-(prop-2-ynyloxy)-phenyl) fulvene was added to a Schlenk flask and was dissolved in 60 mL dry diethyl ether to give a orange solution. The fulvene solution was transferred to the Super Hydride solution *via syringe*. The solution was left to stir for 16 h in which time a pale yellow precipitate of the lithium cyclopentadienide intermediate formed and the solution had changed its colour from orange to clear. The precipitate was filtered onto a frit and was washed with diethyl ether. The pale yellow precipitate was dried briefly under reduced pressure and was transferred to a Schlenk flask under nitrogen. 1.58 g (7.31 mmol, 80% yield) of the lithiated cyclopentadienide intermediate was obtained and dissolved in 60 mL of dry THF to give a clear solution in a Schlenk flask. 3.7 mL (3.7 mmol) of a 1.0 M titanium tetrachloride solution was added directly to the Schlenk flask containing the dissolved intermediate. After 24 h of reflux the solution became dark red, was then cooled and the solvent was removed under reduced pressure. The remaining dark red residue was extracted with 60 mL of dichloromethane and filtered through Celite to remove the remaining LiCl. The red filtrate was filtered twice more by gravity filtration. The solvent was removed under reduced pressure to give a dark brown solid (Yield: 1.39 g, 2.59 mmol, 71%).

^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.49 (s, 2H, OCH_2CCH), 4.03 (s, 4H, C_5H_4–CH_2), 4.66 (s, 4H, OCH_2CCH), 6.30 (m, 8H, C_5H_4), 6.91 (d, 2H, J = 6.9 Hz, C_6H_4), 7.14 (d, 2H, J = 7.1 Hz, C_6H_4) ppm. ^{13}C NMR ($CDCl_3$, 126 MHz, proton decoupled): δ 156.3 (C_q–O of C_6H_4), 137.5 (C2, C5 of C_5H_4), 132.5 (C3, C4 of C_5H_4), 131.3 (C_q–CH_2 of C_6H_4), 130.1 (C1 of C_5H_4), 115.1 (C2, C6 of C_6H_4), 75.5 (OCH_2CCH), 78.60 (OCH_2CCH), 55.9 (OCH_2CCH), 36.1 (C_5H_4–CH_2) ppm. IR absorptions (solid, KBr): 3297, 3115, 2962, 2956, 2923, 2852, 1608, 1578, 1509, 1454, 1432, 1383, 1259, 1240, 1213, 1174, 1097, 1018, 801, 667, 648, 475 cm^{-1}. UV–Vis (CH_2Cl_2, nm): 288 (ε 36338), 263 (ε 29823), 297 (ε 17061), 324 (ε 10,075), 423 (ε 2998), λ_{max} 555 (ε 443). *Anal.* Calc. for $TiC_{30}H_{26}O_2Cl_2$: C, 67.0; H, 4.9; Cl, 13.2. Found: C, 69.0; H, 5.8; Cl, 10.9%.

2.2.3. N_3–CH_2–C(O)–Phe–OMe (**3**)

Open to air, azidoacetic acid (404 mg, 4 mmol) and TBTU (1.28 g, 4 mmol) were stirred in dichloromethane (12 mL) for 15 min. *N*,*N*-diisopropylethylamine (0.68 mL, 28 mmol) was added and the resultant homogenous solution stirred for 10 min. H–Phe–OMe–HCl (0.86 g, 4.0 mmol) was added and the mixture stirred in the dark at room temperature over night. After removal of the volatiles, the remaining oil was dissolved in ethyl acetate (40 mL) and the solution washed with aqueous $KHSO_4$ (1 N, 2 × 40 mL), $NaHSO_4$ (5%, 2 × 50 mL) and brine (2 × 50 mL). The



Scheme 1. Synthesis of the alkyne-substituted fulvene **1**.

organic layer was dried over $MgSO_4$ and evaporated to dryness to give N_3–CH_2–C(O)–Phe–OMe as a colourless solid (Yield: 0.84 g, 3.2 mmol, 80%). An analytically pure sample was obtained by crystallisation from ethylacetate.

^{1}H NMR (250 MHz, $CDCl_3$): *δ 7.33–7.10 (m, 5H, C_6H_5 of Phe), 6.65 (d, J = 7.7, 1H, NHCO), 4.86 (m, 1H, α-CH of Phe), 3.90 (s, 2H, N_3–CH_2), 3.70 (s, 3H, CO_2CH_3) 3.19–3.01 (m, 2H, β-CH_2 of Phe) ppm.* ^{13}*C NMR (62.5 MHz, $CDCl_3$, proton decoupled): δ 171.6 (CO_2CH_3), 166.5 (CONH), 135.7 (C_{q2} of C_6H_5), 129.4 (C3, C5 of C_6H_5), 128.9 (C2, C6 of C_6H_5), 127.5 (C4 of C_6H_5), 53.2 (α-CH of Phe), 52.8 ($COCH_3$), 52.6 (N_3–CH_2), 38.1 (β-CH_2 of Phe) ppm. Anal. Calc. for $C_{12}H_{14}N_4O_3$: C, 54.96; H, 5.38; N, 21.36. Found: C, 55.70; H, 5.29; N, 21.75%.*

*2.2.4. Bis{[2-(4-((1-(2-(1-methoxy-1-oxo-3-phenylpropan-2-ylamino) -2-oxoethyl)-1H-1,2,3-triazol-4-yl)methoxy)-benzyl)]-cyclopentadienyl} titanium(IV) dichloride (**4**)*

Bis-[*p*-(prop-2-ynyloxy)-benzyl-cyclopentadienyl] titanium(IV) dichloride **2** (83 mg, 0.16 mmol), N_3–CH_2–C(O)–Phe–OMe **3** (89 mg, 0.34 mmol) and CuI (6 mg, 0.03 mmol) were mixed and dissolved in CH_2Cl_2/acetonitrile (2:1, 12 mL). After addition of 2,6-lutidine (40 μL, 0.34 mmol) the mixture was degassed, purged with argon and stirred for 12 h in the dark. Removal of the volatiles gave a light brown solid, that was extracted with chloroform (2 × 8 mL). The volume of the orange extract was reduced to ca. 2 mL and Et_2O (20 mL) was added to obtain an orange precipitate, that was separated by filtration, and washed with Et_2O (2 × 4 mL) and hexane (2 × 4 mL). Drying under reduced pressure gave **4** as an orange powder (Yield: 80 mg, 0.075 mmol, 48%).

^{1}H NMR (250 MHz, $CDCl_3$,): δ 7.67 (s, 2H, C*H* of triazole), 7.44 (m, 2H, C_6H_5 of Phe), 7.23–6.87 (m, 16H, C_6H_5 of Phe, C_6H_4), 6.50 (d, 2H, *J* = 7.7, N*H*), 6.25 (m, 8H, C_5H_4–CH_2), 5.19 (s, 4H, C_6H_4–OCH_2), 4.99 (s, 4H, NCH_2–CONH), 4.80 (dd, 2H, *J* = 7.7; 9.2, α-C*H* of Phe), 3.98 (s, 4H, C_5H_4–CH_2), 3.70 (s, 6H, CO_2CH_3), 3.15–3.00 (m, 4H, β-CH_2 of Phe) ppm. ^{13}C NMR (62.5 MHz, $CDCl_3$, proton decoupled): *Note:* Signal for O–CH_2–triazole is obscured by the solvent signal. δ 171.5 (CO_2CH_3), 164.8 (CONH), 157.0 (C_q–O of C_6H_4), 144.9 (C_q of triazole), 137.9 (C2, C5 of C_5H_4), 135.4 (C3, C4 of C_5H_4), 131.7 (C1 of C_5H_4), 135.6 (C_q of C_6H_5), 130.4 (C3, C5 of C_6H_4), 129.3 (C3, C5 of C_6H_5), 128.9 (C2, C6 of C_6H_5), 127.5 (CH_2-C_q of C_6H_4, C_6H_5), 115.1 (C2, C6 of C_6H_4), 122.2 (CH of triazole), 62.1 (N–CH_2–CO), 53.6 (α-CH of Phe), 52.8 (OCH_3), 37.8 (β-CH_2 of Phe), 36.2 (C_5H_4-CH_2) ppm. *Anal.* Calc. for $C_{54}H_{54}Cl_2N_8O_8Ti$: C, 61.08; H, 5.13; N, 10.55. Found: C, 60.98; H, 5.45; N, 7.92%. ESI-MS (pos. mode): *m*/*z* 1021.24 $[M–2Cl+CH_3O^-]^+$, 495.28 ($[Cp^R+H+Na]^+$), 473.32 ($[Cp^R+2H]^+$) (exact Mass for $C_{54}H_{54}Cl_2N_8O_8Ti$ = 1060.29).

3. Results and discussion

The synthesis of the alkyne-substituted fulvene **1** from the corresponding benzaldehyde derivative followed the conditions outlined by Stone and Little [16] employing pyrrolidine as a catalyst in methanol. The synthesis, which is shown in Scheme 1, gave **1** in 66% yield after purification. The fulvene was characterised and purified by column chromatography, then carried onto the titanocene synthesis.

Titanocene **2** was synthesised via the hydridolithiation procedure developed in the Tacke group [3]. This route uses $LiBEt_3H$ in diethyl ether to transfer a hydride to a fulvene through nucleophilic addition to the exocyclic double bond of the fulvene **1** to form an insoluble lithium cyclopentadienide intermediate. This precipitated intermediate can then be washed on a glass frit, dried and weighed to be used stoichiometrically in the next reaction step. Two molar equivalents of the lithiated intermediate underwent a transmetallation reaction in each case when reacted with one molar equivalent of $TiCl_4$ in THF under reflux, to give the alkyne-substituted titanocene **2**. This reaction proceeded smoothly with the alkyne-substituted fulvene **1** to give an overall yield of 57% for both steps and is shown in Scheme 2.

Table 2
Selected bond lengths and angles of titanocene **2**.

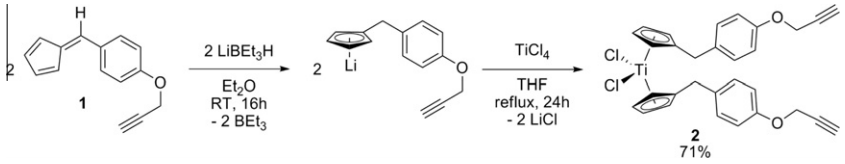
Bond length (Å)	
Ti-Cent	2.062(1)
Ti–Cl	2.367(3)
C(14)–C(15)	1.185(2)
Bond angle (°)	
Cl–Ti–Cl#1	94.84(2)
Cent-Ti-Cent#1	132.31(1)

Symmetry transformations used to generate equivalent atoms: #1 $-x, y, -z + 1/2$.

3.1. Structural discussion

For the purpose of X-ray diffraction, suitable single crystals of **2** were grown by vapour diffusion of pentane into a saturated solution of the compound in dichloromethane. The collection and refinement data for the crystal is shown in Table 1. The determined structure for the alkyne-substituted titanocene dichloride derivative showed rod-like molecules packed in the solid state. Selected bond lengths and angles are shown in Table 2.

The titanocene dichloride derivative **2**, which is shown in Fig. 1, crystallises in the space group C2/c with the titanium centre placed on the twofold axis. Titanocene **2** has a distorted tetrahedral shape with respect to its two halogenido ligands and two cyclopentadienide groups. The titanium–cyclopentadienide centroid distance was determined as 2.062(1) Å, while the titanium–chlorine bond length was found to be 2.367(3) Å. The centroid-to-titanium-to-centroid angle was measured to be 132.31(1)°, which is a significant deviation from a normal tetrahedral angle due to the relative bulk of the substituted cyclopentadienide groups. The Cl–Ti–Cl bond angle on the other side was squeezed to 94.84(2)°, which again is a reflection on the bulky Cp ligands. All these values compare very well to the corresponding ones in Titanocene Y [3]. Titanocene **2** is slightly more rod shaped than Titanocene Y, which appears more Z-shaped in its packing. There are no π–π interactions to be seen between the substituted phenyl groups in any of the compounds and no solvent molecules are needed to stabilise



Scheme 2. Synthesis of the alkyne-substituted titanocene dichloride derivative **2**.

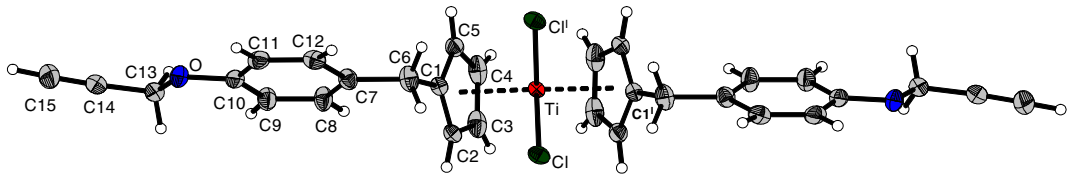
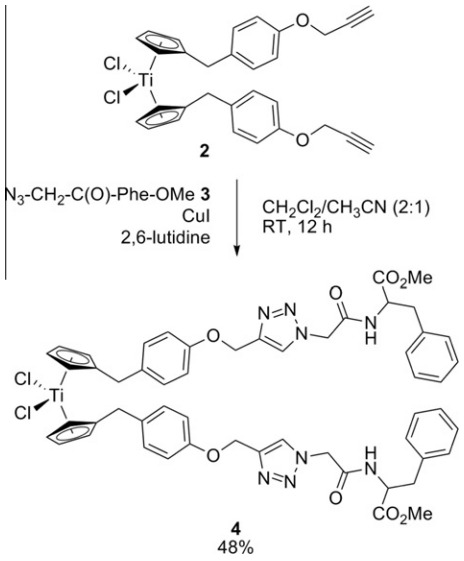


Fig. 1. Molecular structure of **2**; thermal ellipsoids are drawn on the 50% probability level; symmetry operation: $^{I}-x, y, -z + 1/2$.



Scheme 3. Synthesis of triazole-conjugate **4** by CuAAC.

the lattice of **2**. The carbon–carbon triple bond between C14 and C15 was found to be 1.185(2) Å in length and delivers the basis for the reactivity of the metallocene in the copper-catalysed azide–alkyne cycloaddition.

3.2. Cu-catalysed [3+2] cycloaddition

In order to test the suitability of alkyne-substituted titanocene dichloride derivatives in Cu-catalysed [3+2] cycloadditions, **2** was coupled to azide-functionalised, methylester-protected phenylalanine **3** as outlined in Scheme 3. To avoid the use of water, the reaction was performed in a dichloromethane/acetonitrile mixture using copper iodide in the presence of 2,6-lutidine [12]. Formation of the triazole compound **4** was monitored by decrease of the ^{1}H NMR signal of the alkynyl-function and appearance of a singlet for the triazole hydrogen (7.67 ppm in $CDCl_3$). Although characterisation of **4** by ^{1}H as well as ^{13}C NMR indicated no impurities, elemental analysis repeatedly gave low values for nitrogen, presumably due to formation of titanium nitride during sample combustion. Analysis of **4** by ESI-MS (pos. mode) showed one major signal with a titanium isotope pattern ($m/z = 1021.24$), corresponding to loss of two chloride ligands and addition of a methoxide ion. Two additional signals at $m/z = 495.28$ ($[Cp^{R}+H+Na]^{+}$) and 473.32 ($[Cp^{R}+2H]^{+}$) indicate cleavage of the functionalised Cp ligands.

Attempts to couple azide-functionalised enkephalin to the alkyne-substituted titanocene dichloride derivative **2** were unsuccessful, likely due to insufficient solubility of the peptide as well as complexation of the copper catalyst by the peptide-carboxy function in CH_2Cl_2/CH_3CN. Attempts to apply the common H_2O/*t*BuOH system with $CuSO_4$ as a catalyst resulted in rapid degradation of the titanocene **2** and formation of insoluble species.

4. Conclusions and outlook

A first step towards peptide-substituted and therefore targeted titanocene dichloride derivatives was performed. It is principally possible to react an alkyne-substituted metallocene dihalogenide compound with a low molecular weight azide-functionalised protein mimic under CuAAC conditions employing a dichloromethane/acetonitrile 2:1 solvent mixture that prevents hydrolysis of the dihalogenide ligands. Unfortunately but not unexpectedly, this experimental procedure could not be extended to the oligopeptide enkephalin, which made the synthesis of a peptide-substituted titanocene dichloride derivative starting from **2** impossible. In the future, a new titanocene derivative carrying the alkynyl-substituent as an anion will be synthesised and reacted with azide-functionalised enkephalin to reach the goal of a peptide-substituted titanocene anticancer drug.

Acknowledgements

The authors thank the Higher Education Authority (HEA), the Centre for Synthesis and Chemical Biology (CSCB), the Irish Research Council for Science, Engineering and Technology (IRCSET), University College Dublin (UCD) and COST D39 for funding. J.Z. is a fellow of RUB research school, while D.W. received a postgraduate studentship from IRCSET.

Appendix A. Supplementary data

CCDC 814189 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html, or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk.

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