

Metallo-carbonyl conjugates of biotin and biocytin

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Abstract

Coupling of biotin with $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N(1)\text{-}4\text{-aminosulfonamido})$ (**1a**) and $\text{CpFe}(\text{CO})(\text{P}(\text{OMe})_3)(\eta^1\text{-}N(1)\text{-}4\text{-aminosulfonamide})$ (**1b**) afforded corresponding metallo-carbonyl biotin amides **2a–b**. Reaction of N_ϵ -biotinyl-L-lysine (biocytin) with $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N(1)\text{-}3\text{-isothiocyanatophthalimidato})$ (**4a**), $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N(1)\text{-}4\text{-isothiocyanatophthalimidato})$ (**4b**), $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N(1)\text{-}4\text{-isothiocyanatosulfonamido})$ (**5**, this compound was prepared from **1a** and thiophosgene and its structure confirmed by spectral data and by the X-ray diffraction method) gave bioconjugates **6a–c**. The HABA-avidin test revealed high affinities of all synthesised bioconjugates to avidin.

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1. Introduction

The extraordinarily strong affinity of biotin to (strept)avidin ($K_m = 10^{14}\text{--}10^{15} \text{ M}^{-1}$) has found widespread applications in biochemical research, medical diagnosis and radioimmunotherapy [1–3]. Bioanalytical applications of the biotin-(strept)avidin system require biotin conjugates containing various reporter groups used as markers in competitive assays. IR-detectable metallo-carbonyl markers constitute an new alternative to frequently used fluorescent ones [4–7]. Their sensitive detection is based on the presence of very strong absorption bands due to the stretching vibrations of co-ordinated CO ligands in the region $1850\text{--}2100 \text{ cm}^{-1}$, which is virtually free from any absorption originating from biomolecules or biological matrices. Metallo-carbonyl markers have been successfully applied in a study of hormone-receptor interactions and immunoassays (carbonylmetalloimmunoassay). We have recently reported labelling of biotin with metal-carbonyl moieties such as dicobalt hexacarbonyl, cyclopentadienyl man-

ganese tricarbonyl and cyclopentadienyl iron dicarbonyl ($\eta^1\text{-}N\text{-succiminato}$) [8]. Multiple labelling was achieved by attachment of both biotin and metal carbonyl fragments to a protein or a dendrimer core. Herein, we report the synthesis of metallo-carbonyl conjugates of biotin, and its derivative, biocytin (N_ϵ -biotinyl-L-lysine), based on the $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N\text{-sulfonamide})$ and $\text{CpFe}(\text{CO})_2(\eta^1\text{-}N\text{-phtalimidato})$ systems described earlier [9,10], that are of interest for developing a IR-detectable biotinylated probe for biochemical applications.

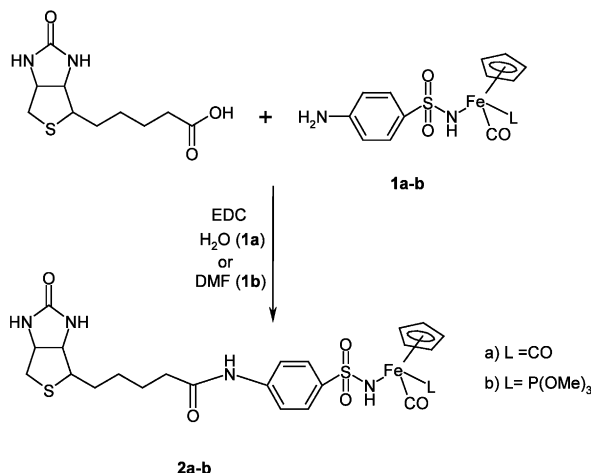
2. Results and discussion

2.1. Metallo-carbonyl conjugates of biotin

We have recently described the synthesis of the complex, $(\eta^5\text{-C}_5\text{H}_5)\text{Fe}(\text{CO})_2[\eta^1\text{-}N(4\text{-aminobenzenesulfonamido})]$, (**1a**), and acylation of its primary amino function with carboxylic acids in the presence of 1-ethyl-3-[3-(dimethylamino)]carbodiimide hydrochloride (EDC) [9]. We applied this reaction for coupling of **1a** with biotin (Scheme 1). Since **1a** is sparingly soluble in water the reaction was carried out in this solvent. The

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Scheme 1.

product **2a** precipitates directly from the reaction mixture in 57% yield.

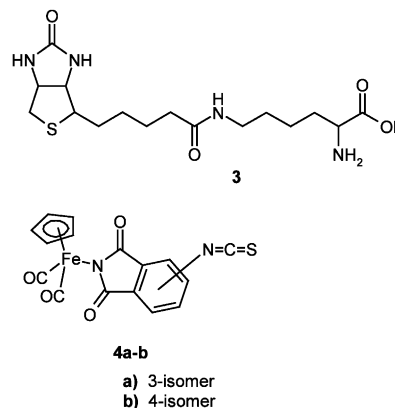
The IR spectrum of **2a** exhibits two strong absorption bands characteristic of the (η^5 -C₅H₅) Fe(CO)₂ moiety at 2050 and 2005 cm⁻¹. As the positions and number of ν (CO) bands is critical for the eventual use of this complex in quantitative bioassays, we attempted to transform **2a** into a monocarbonyl complex, **2b**, for which only one ν (CO) band was expected at ~1980 cm⁻¹. We attempted to obtain **2b** by refluxing **2a** with trimethylphosphite in ethanol or by photolysing this complex in DMF in the presence of trimethylphosphite, but all our attempts failed. We have, therefore decided to try to exchange one CO ligand in **1a** by trimethylphosphite to obtain **1b** and then couple this complex with biotin.

We have found that **1a** can be transformed in modest yield (34%) into **1b**, by photolysis in DMF containing a fourfold excess of trimethylphosphite. **1b** was characterized by IR, ¹H-NMR and elemental analysis. The coupling of **1b** with biotin was carried out in DMF using EDC as the carboxyl group activator. The product **2b** was isolated in ~30% yield (59% based on reacted **1b**). Conjugates **2a–b** gave correct CHNS analysis assuming molecular formulae **2a**·H₂O and **2b**·4H₂O. Their FAB mass spectra (nitrobenzyl alcohol matrix) displayed peaks corresponding to M+H⁺ and M-H⁻ ions in the positive and negative ions mode, respectively. Interestingly, in the spectrum of **2b** the peak corresponding to *m/e* 742 (M+4H₂O) was observed in both modes confirming suggested composition and revealing exceptionally tight water binding. As expected, IR spectrum of **2b** (KBr) displayed the very strong absorption band at 1965 cm⁻¹ due to the stretching vibrations of the CO ligand.

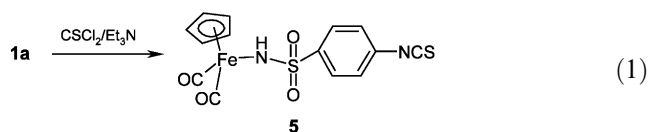
2.2. Metallo-carbonyl conjugates of biocytin

Biocytin (*N*_ε-biotinyl-L-lysine) **3** is a commercially available biotin derivative containing both carboxylic and primary amino function. We thought that it would be of interest to attach the metallo-carbonyl moiety to the amino function of **3** because the presence in such conjugates of the carboxylic group will allow their attachment to biomolecules. We have decided to use for labelling of **3** organometallic isothiocyanates **4a–b** [10].

Formulae 3 and 4a–b



We have also synthesised a new metallo-carbonyl isothiocyanate **5** via reaction of **1a** with thiophosgene in the presence of triethylamine in DMF (Eq. (1)).



The isothiocyanate **5** was isolated in 76% yield and its structure confirmed by IR, ¹H-NMR and elemental analysis. The molecular structure of **5** was also confirmed by the X-ray diffraction method. The crystal data are gathered in Table 1, selected bonds lengths and angles in Table 2. The view of the molecule of **5** is shown in Fig. 1.

The reaction of **3** with isothiocyanates **4a–b** and **5** was carried out in DMF–water in the presence of an excess of triethylamine (Scheme 2).

The conjugates **6a–c** were isolated as orange air-stable microcrystalline solids in 82–94% yield and their structures were confirmed by IR, ¹H-NMR elemental analyses and FAB MS. They are soluble in aqueous Na₂CO₃ and precipitate from such solutions on acidification with diluted HCl.

2.3. Affinity of 2a–b and 6a–c to avidin

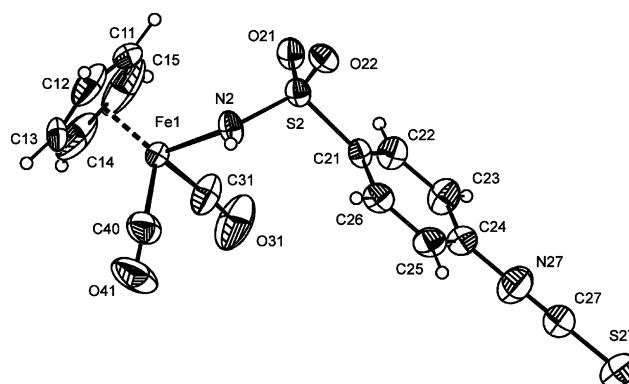
To assess the affinity of **2a–b** and **6a–c** to avidin we have used the HABA-Avidin reagent (Sigma). The

Table 1
Crystallographic data

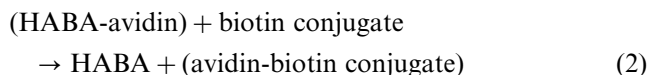
Molecular formula	C ₁₄ H ₁₀ FeN ₂ O ₄ S ₂
Molecular weight	390.21
Crystal description and size (mm)	Orange cubic block 0.25 × 0.25 × 0.3
Crystal system	Triclinic
Space group	$P\bar{1}$
<i>a</i> (Å)	8.544(1)
<i>b</i> (Å)	12.953(1)
<i>c</i> (Å)	7.617(1)
α (°)	94.51(1)
β (°)	93.80(1)
γ (°)	101.70(1)
<i>V</i> (Å ³)	820.0(1)
<i>Z</i>	2
<i>d</i> _x (g cm ^{−3})	1.580
<i>F</i> (000)	396
μ (cm ^{−1})	99.43
<i>T</i> (K)	293(2)
Max θ (°)	72.69
<i>hkl</i> ranges	−10.9; −16.15; −9.9
No. of reflns measd	3345
No. of indep reflns	3114
<i>R</i> _{int}	0.030
Refinement type	<i>F</i> ²
Hydrogen atoms	Mixed
No. of params refined	205
Reflns/params	15
<i>R</i> (<i>F</i>) ^a	0.0983
<i>wR</i> (<i>F</i> ²) ^b	0.1193 ^c
<i>R</i> (<i>F</i>) ^a	0.0437 for 1663 reflections with <i>I</i> > 2 σ (<i>I</i>)
<i>wR</i> (<i>F</i> ²) ^b	0.0839 for 1663 reflections with <i>I</i> > 2 σ (<i>I</i>) ^c
GOF	0.938
Difference peak/hole (e Å ^{−3})	0.269/−0.452

^a $R(F) = \Sigma(|F_o - F_c|) / \Sigma|F_o|$.^b $wR(F^2) = [\Sigma w(|F_o - F_c|)^2 / \Sigma |F_o|^2]^{1/2}$.^c $w = (\exp[1.3(\sin \theta / \lambda)^2] / [\sigma^2(F_o^2) + (0.0338P)^2])$ where $P = [(F_o^2) + 2(F_c^2)]/3$.Table 2
Selected bond lengths (Å) and angles (°) in **5**

Bond lengths	
Fe ₁ –N ₂	1.965(4)
Fe ₁ –C ₁₁	2.071(6)
Fe ₁ –C ₁₂	2.076(6)
Fe ₁ –C ₁₃	2.052(8)
Fe ₁ –C ₁₄	2.045(8)
Fe ₁ –C ₁₅	2.033(6)
Fe ₁ –C ₃₀	1.750(6)
Fe ₁ –C ₄₀	1.770(6)
S ₂ –N ₂	1.565(4)
S ₂ –O ₂₁	1.430(3)
S ₂ –O ₂₂	1.451(3)
Bond angles	
N ₂ –S ₂ –O ₂₁	110.2(2)
N ₂ –S ₂ –O ₂₂	109.7(2)
N ₂ –S ₂ –C ₂₁	109.7(2)
C ₂₁ –S ₂ –O ₂₁	105.8(2)
C ₂₁ –S ₂ –O ₂₂	104.0(2)
O ₂₁ –S ₂ –O ₂₂	117.0(2)
Fe ₁ –N ₂ –S ₂	129.8(2)
C ₃₀ –Fe ₁ –N ₂	96.4(5)
C ₄₀ –Fe ₁ –N ₂	91.2(2)
C ₃₀ –Fe ₁ –N ₂	94.2(3)

Fig. 1. The view of the molecule of **5** with atom-numbering scheme. Displacement ellipsoids are shown at 30% probability level.

formation the complex (avidin–biotin conjugate) was monitored by disappearance of the absorbance at 500 nm, characteristic for the HABA–avidin complex according to the Eq. (2).



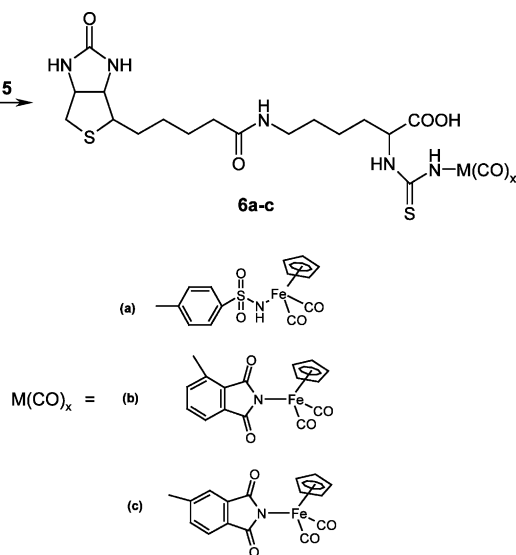
We have found that changes caused by addition **2a–b** or **6a–c** are practically the same as those caused by addition of the equivalent amount of biotin. For example, Fig. 2 shows plots of the changes of absorbance at 500 nm against concentration of **6a–b** (or biotin).

This reveals high affinity of synthesised conjugates to avidin and their applicability for assays based on the

biotin–avidin system at least at micromolar concentrations.

3. Experimental

All reactions were carried out under argon. Chromatographic separations were carried out using silica gel 60 (Merck, 230–400 mesh ASTM) ¹H-NMR spectra were recorded on a Varian Gemini 200BB (200 MHz) spectrometer. IR spectra were recorded on a Biorad spectrometer. Mass spectra were recorded on a Finnigan MAT 95 spectrometer. Compounds **4a–b** and **5** were prepared according to earlier published procedures [9,10]. Other reagents were commercially available and used without prior purification.



Scheme 2.

3.1. Synthesis of 2a

A solution of **1a** (150 mg, 0.43 mmol), biotin (70 mg, 0.29 mmol) and EDC (220 mg, 1.15 mmol) in water (30 ml) was stirred 3 h at r.t. The orange precipitate was filtered, washed with water, ether and dried. Yield: 94 mg (57%).

¹H-NMR (dms-*d*₆, δ ppm): 10.09 (s, 1H, NH amide); 7.75 (s, 1H, sulfonamide); 7.76 (d, J = 8.0 Hz, 2H) and 7.56 (d, J = 8.0 Hz, 2H) aromatic H's; 6.46 (s, 1H, NH biotin); 6.38 (s, 1H, NH biotin); 5.15 (s, 5H, Cp); 4.30 (m, 1H) and 4.15 (m, 1H), CH (biotin); 2.34 (m, 2H, CH₂CO); 1.6–1.4 (m, 6H, other CH₂). IR (KBr, cm⁻¹): 3324 (NH); 2036, 1994 (Fe–CO); 1699 (CO amide + biotin); 1531 (NH amide); 1307 and 1130 (SO₂). FAB MS (nitrobenzyl alcohol matrix, *m/e*): positive ions mode: 575 [M+H]⁺, 519 [M+H–2CO]⁺; negative ions mode: 573 [M+H]⁺, 517 [M+H–2CO]⁺.

H–2CO]⁺. Anal. Found: C, 46.61; H, 4.79; N, 9.44; S, 10.53. Calc. For C₂₃H₂₈FeN₄O₇S₂ (monohydrate): C, 46.63; H, 4.76; N, 9.46; S, 10.82.

3.2. Synthesis of 1b

A solution of **1a** (160 mg, 0.46 mmol) and trimethylphosphite (0.217 ml, 1.84 mmol) in DMF (5 ml) was irradiated with visible light (a setup of 4 × 150 W tungsten lamps) at 0 °C for 2 h. After evaporation to dryness the residue was chromatographed collecting the red fraction eluted with chloroform–methanol (9:1). Crystallization (dichloromethane–ether) afforded pure **2b**. Yield: 77 mg (38%). ¹H-NMR (dms-*d*₆, δ ppm): 7.39 (d, J = 8.0 Hz, 2H, H_{ar}), 6.58 (d, J = 8.0 Hz, 2H, H_{ar}), 5.50 (s, 2H, NH₂), 4.44 (s, 5H, Cp), 3.65 (d, J = 11.1 Hz, 9H, OMe). IR (KBr, cm⁻¹): 3428, 3342, 3251 (NH + NH₂), 1957 (Fe–CO), 1284, 1122 (SO₂), 1020, 748 (PO). Anal. Found: C, 40.30; H, 4.74; N, 6.61. Calc. For C₁₅H₂₁FeN₂O₆PS₂: C, 40.56; H, 4.76; N, 6.31.

3.3. Synthesis of 2b

A solution of **1b** (80 mg, 0.18 mmol), biotin (44 mg, 0.18 mmol) and EDC (138 mg, 0.72 mmol) in DMF (3 ml) was stirred overnight at r.t. The solvent was evaporated and the residue chromatographed (chloroform–methanol 9:1 as eluent). Two red bands were collected, the first being unreacted **1b** and the second **2b**. Crystallization from dichloromethane–ether afforded analytically pure sample of **2b**. Yield: 36 mg (30%).

¹H-NMR (dms-*d*₆, δ ppm): 10.13 (s, 1H, NH amide), 7.70 (m, 4H, H_{ar}), 6.45 (s, 1H, NH biotin), 6.38 (s, 1H, NH biotin), 4.43 (s, 5H, Cp), 4.31 (m, 1H, CH biotin), 4.16 (m, 1H, CH biotin); 2.34–3.65 (d, J = 10.0 Hz, 9H, MeO). 2.35 (m, 2H, CH₂CO); 1.6–1.4 (m, 6H, other CH₂). IR (KBr, cm⁻¹): 3295, 3195, 3090

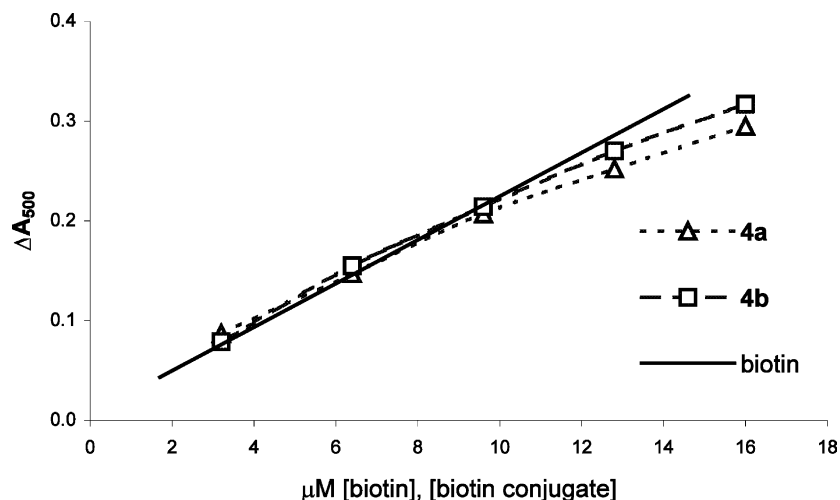


Fig. 2. Plot of the changes of absorbance at 500 nm of the HABA-Avidin complex against concentration of biotin or biotin conjugates (**4a–b**).

(NH), 1965 (Fe–CO); 1694 (CO amide+biotin); 1534 (NH amide); 1311, 1131 (SO₂), 1021, 751 (PO). FAB MS (nba matrix *m/e*): positive ions mode: 742 [M+H+4H₂O]⁺, 671 [M+H]⁺; negative ions mode: 742 [M+H+4H₂O][−], 669 [M−H][−], 517. Anal. Found: C, 40.56; H, 5.74; N, 7.65. Calc. For C₂₅H₄₃FeN₄O₁₂PS₂ (tetrahydrate): C, 40.44; H, 5.74; N, 7.56.

3.4. Synthesis of **5**

To a solution of **1a** (522 mg, 1.5 mmol) in DMF (5 ml) containing triethylamine (0.3 ml) thiophosgen (0.15 ml, 1.96 mmol) was added at 0–5 °C and the mixture was stirred at temperature 0.5 h. The precipitated triethylamine hydrochloride was filtered off and the solvent evaporated. The residue was dissolved in chloroform (5 ml) and washed twice with water. After evaporation and column chromatography (eluent chloroform) and crystallization (dichloromethane–hexane) the red **5** was obtained. Yield: 444 mg (76%).

¹H-NMR (dms-*d*₆, δ ppm): 7.84 (s, 1H, NH), 7.68 (d, *J* = 6.0 Hz, 2H, H_{ar}), 7.52 (d, *J* = 6.0 Hz, 2H, H_{ar}), 5.15 (s, 5H, Cp). IR (KBr, cm^{−1}): 3254 (NH), 2109 (NCS) 2039, 1991 (Fe–CO).

3.5. Coupling of **4a–b** and **5** with biocytin

Biocytin (30 mg, 0.08 mmol) and the organometallic isothiocyanate **4a–b** or **5** (0.1 mmol) were dissolved in DMF (2.1 ml), water (0.5 ml) and triethylamine (0.5 ml) and stirred 2 h at r.t. After evaporation to dryness the residue was dissolved in water (~5 ml). The products precipitate on acidification to pH 3 with 0.6 M HCl.

6a. Yield: 94%. ¹H-NMR (dms-*d*₆, δ ppm): 9.97 (s, 1H, NH), 9.35 (b, 1H, NH), 8.67 (d, *J* = 7.7 Hz, 1H, H_{ar}), 7.8 (b, 1H, NH), 7.46 (t, *J* = 7.7 Hz, H_{ar}), 7.21 (d, *J* = 7.7 Hz, 1H, H_{ar}), 6.43 (s, 1H, NH biotin), 6.38 (s, 1H, NH biotin), 5.38 (s, 5H, Cp), 4.30 (m, 1H, CH biotin), 4.12 (m, 1H, CH biotin); 2.1 (m, 4H), 1.4 (m, 12H). IR (KBr, cm^{−1}): 2047, 1998 (Fe–CO). FAB MS (nba matrix *m/e*): negative ions mode: 751 [M−H][−], 695 [M−H−2CO][−]. Anal. Found: C, 51.39; H, 4.49; N, 10.05. Calc. For C₃₂H₃₆FeN₆O₈S₂: C, 51.07; H, 4.82; N, 11.17.

6b. Yield: 93%. ¹H-NMR (dms-*d*₆, δ ppm): 10.09 (s, 1H, NH), 8.20 (d, *J* = 7.6 Hz, 1H, NH), 7.98 (s, 1H, H_{ar}), 7.80 (b, 1H, NH), 7.56 (d, *J* = 7.6 Hz, 1H, H_{ar}), 7.45 (d, *J* = 7.6 Hz, 1H, H_{ar}), 6.45 (s, 1H, NH biotin), 6.35 (s, 1H, NH biotin), 5.34 (s, 5H, Cp), 4.30 (m, 1H, CH biotin), 4.12 (m, 1H, CH biotin); 2.1 (m, 4H), 1.4 (m, 12H). IR (KBr, cm^{−1}): 2046, 1997 (Fe–CO). FAB MS (nba matrix *m/e*): negative ions mode: 751 [M−H][−], 695 [M−H−2CO][−]. Anal. Found: C, 49.87; H, 4.85; N, 10.61; S, 8.13. Calc. For C₃₂H₃₈FeN₆O₉S₂ (monohydrate): C, 49.84; H, 4.67; N, 10.90; S, 8.31.

6c. Yield: 62%. ¹H-NMR (dms-*d*₆, δ ppm): 10.04 (s, 2H, NH amide+COOH), 8.3–7.4 (m, 7H, H_{ar}+NH), 7.70 (m, 4H, H_{ar}), 6.4 (b, 2H, NH biotin), 5.17 (s, 5H, Cp), 4.31 (m, 1H, CH biotin), 4.13 (m, 1H, CH biotin); 2.1 (b, 4H), 1.4 (m, 12H). IR (KBr, cm^{−1}): 3295, 3090 (NH), 2046, 1997 (Fe–CO). FAB MS (nba matrix *m/e*): positive ions mode: negative ions mode: 761 [M−H][−], 733 [M−H−CO][−], 705 [M−H−2CO][−]. Anal. Found: C, 44.08; H, 5.20; N, 9.90; S, 11.87. Calc. For C₃₀H₄₂FeN₆O₁₁S₃ (trihydrate): C, 44.11; H, 5.43; N, 10.29; S, 11.75.

3.6. X-ray structure determination

Data were collected on a Rigaku AFC5S diffractometer using Cu Kα (λ = 1.54178 Å) X-ray source and a graphite monochromator. Analytical absorption corrections was applied. Experimental details are given in the Table 1. The crystal structure was solved by direct methods using SHELXS86 [11] and refined by full-matrix least square method using SHELXL97 [12]. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms except for H₂ atom were introduced in the calculated positions and refined using the rigid body model. The position of the H₂ atom, taking part in an intermolecular hydrogen bonding with O₂₂ of the other molecule, was found on the Fourier-difference map and refined freely. The molecular geometry was calculated by PARST [13] and PLATON [14].

4. Supplementary material

Crystallographic data for the structural analysis have been deposited with the Cambridge Crystallographic Data Centre, CCDC No. 194449 for compound **5**. Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (Fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or <http://www.ccdc.cam.ac.uk>).

Acknowledgements

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References

- [1] M. Aslam, A. Dent, Bioconjugation (Chapter 5), MacMillan Reference Ltd, London, 1998.

- [2] G.T. Hermanson, *Bioconjugate Techniques* (Chapter 13), Academic Press, San Diego, 1996.
- [3] M. Wilchek, E.A. Bayer, *Anal. Biochem.* 171 (1988) 1.
- [4] M. Salmain, A. Vessieres, G. Jaouen, *Anal. Chem.* (1991) 63.
- [5] G. Jaouen, A. Vessieres, I.S. Butler, *Acc. Chem. Res.* 26 (1993) 361.
- [6] A. Varenne, A. Vessieres, M. Salmain, S. Durand, P. Brossier, G. Jaouen, *Anal. Biochem.* 242 (1996) 172.
- [7] A. Vessieres, M. Salmain, P. Brossier, G. Jaouen, *J. Pharm. Biomed. Anal.* 21 (1999) 625.
- [8] M. Salmain, N. Fischer-Durand, L. Cavalier, B. Rudolf, J. Zakrzewski, *Bioconjugate Chem.* 13 (2002) 693.
- [9] M. Pawlak, J. Zakrzewski, *J. Organomet. Chem.* 568 (1998) 171.
- [10] A. Kazimierzczak, J. Zakrzewski, M. Salmain, G. Jaouen, *Bioconjugate Chem.* 8 (1997) 484.
- [11] G.M. Sheldrick, *SHELXS86* Program for Crystal Structure Solution, University of Göttingen, Göttingen, Germany, 1990.
- [12] G.M. Sheldrick, *SHELXL97* Program for the Refinement of Crystal Structures, University of Göttingen, Göttingen, Germany, 1997.
- [13] M. Nardelli, *Comput. Chem.* 7 (1983) 95.
- [14] A.L. Spek, *PLATON* and *PLUTON* Programs for Molecular Geometry Calculation, University of Utrecht, Utrecht, The Netherlands, 1990.