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PAPER

Biotin as acylating agent in the Friedel–Crafts reaction. Avidin affinity of biotinyl derivatives of ferrocene, ruthenocene and pyrene and fluorescence properties of 1-biotinylpyrene

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(D)-Biotin was used for Friedel–Crafts acylation of electron-rich aromatic molecules - ferrocene, ruthenocene and pyrene. The reaction carried out in the presence of trifluoroacetic anhydride and trifluoromethanesulfonic acid afforded the corresponding biotinylarenes in moderate yields. These compounds, although lacking an amide bond, exhibited high affinity for avidin, with the ability to displace 2-(4′-hydroxyphenylazo)-benzoic acid (HABA) in its complex with avidin. Their affinity for avidin was determined by a solid-phase competitive enzymatic assay, which gave IC_{50} values in the range of 33–58 nM (under the same conditions biotin showed $IC_{50} = 24 \pm 7$ nM). 1-Biotinylpyrene (**1c**) excited at 355 nm displayed fluorescence emission in aqueous solutions with $\lambda_{max} = 461$ nm. The fluorescence maximum was shifted to 425 nm upon binding of **1c** to avidin. Formation of the avidin-**1c** complex was also evidenced by quenching of the fluorescence from the protein tryptophan residues (342 nm) and appearance of the emission band of the avidin-bound **1c** at 430 nm as a result of a Förster resonance energy transfer (FRET) phenomenon.

1. Introduction

The biotin-(strept)avidin system, based on the extremely high affinity of biotin and biotinylated molecules for the proteins avidin or streptavidin,[1] is widely used in bioanalysis,[2] construction of artificial metalloenzymes,[3] drug delivery[4] and materials science.[5] The biotin moiety is most frequently attached to biomolecules, spacers or reporter groups using C–N bond forming reactions (amido coupling or formation of hydrazones).[6] The conjugates obtained in this way display relatively high affinity to (strept)avidin (although usually markedly lower than the affinity of parent biotin, $K_d \sim 10^{-13}$–10^{-15} M).[1] There are also a few examples of biotinylation *via* C–C bond formation (Heck and Sonogashira coupling) but this approach requires prefunctionalized (iodinated) substrates.[7]

Herein we report a conceptually novel approach to the biotinylation of electron-rich reporter molecules such as redox-active ferrocene and ruthenocene and luminescent pyrene, based on their Friedel–Crafts acylation with (D)-biotin. The Friedel–Crafts acylation reaction is one of the most potent C–C bond forming reactions of aromatic systems and recent studies showed that it can be performed under relatively mild, "green" conditions.[8] This prompted us to look whether it would be possible to apply this reaction to biotin, which is a derivative of valeric acid, bearing the complex bicyclic moiety (7-oxo-3-thia-6,8-diazabicyclo[3.3.0]oct-4-yl) at the end of the side chain. We found out that the Friedel–Crafts biotinylation of the aforementioned molecules proceeded in moderate yields, and the conjugates formed, although lacking the amide –C(═O)–N–H group, displayed high affinity to avidin. Moreover, the fluorescence emission of the pyrene derivative appeared to be sensitive to the binding of this molecule to avidin. This property was successfully exploited in a liquid-phase, competitive fluorescent assay of biotin.

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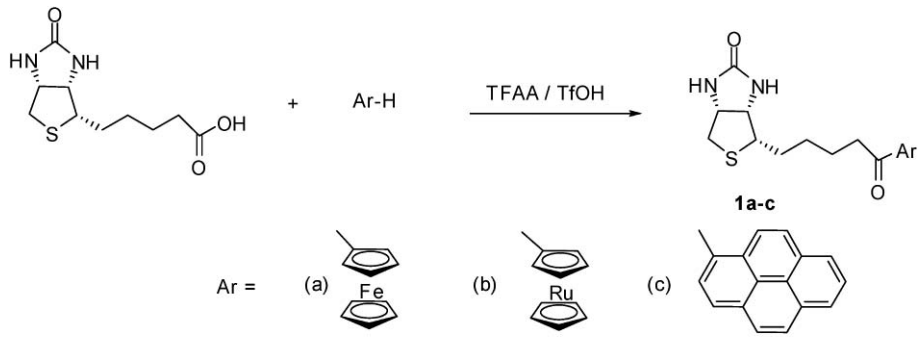
2. Results and discussion

2.1. Friedel–Crafts biotinylation of ferrocene, ruthenocene and pyrene

We reported earlier a mild method of acylation of ferrocene using acyl trifluoroacetates generated *in situ* from carboxylic acids and trifluoroacetic anhydride (TFAA).[9] Herein this method has been applied to the acylation of electron-rich aromatic compounds, namely ferrocene, ruthenocene and pyrene, with (D)-biotin, which is a complex derivative of valeric acid (Scheme 1). The arenes chosen for this study have proven very reactive in Friedel–Crafts acylation (*e.g.* ferrocene and pyrene are 3.3×10^6 and 220 times more reactive than benzene, respectively[10,11]), which offered an opportunity for performing their reaction with biotin under mild conditions, allowing a chance for preservation of integrity of the biotin hetero-bicyclic moiety. To our delight, we found out that they undergo Friedel–Crafts biotinylation upon treatment with



Compound	Yield [%]
1a	55
1b	53
1c	47

Scheme 1 Friedel–Crafts biotinylation of ferrocene, ruthenocene and pyrene.

an equimolar mixture of (D)-biotin and TFAA and 1 equiv. of trifluoromethanesulfonic acid (TfOH) in dichloromethane at r.t. The biotinylketones **1a–c** were isolated in moderate yields and were fully characterized by spectroscopic methods and elemental analyses.

In the case of pyrene we found that the biotinyl group was regioselectively introduced, as expected,[11] at the 1 position. Indeed, the ^{13}C NMR spectrum of **1c** displayed signals of 16 nonequivalent aromatic carbons, characteristic for such a substitution.

To the best of our knowledge compounds **1a–c** represent a novel class of biotin derivatives, in which the biotin entity is bound to an aromatic system through a C–C bond formed by the C═O group. It is worth noting that these compounds should be resistant to the endogenous enzyme biotinidase, which cleaves CO–NH bonds in biotin conjugates and hampers *in vivo* applications of biotin amides.[12] Another interesting feature of compounds **1a–c** is the presence of the *ketone* C═O function which offers numerous possibilities of further structural transformations.

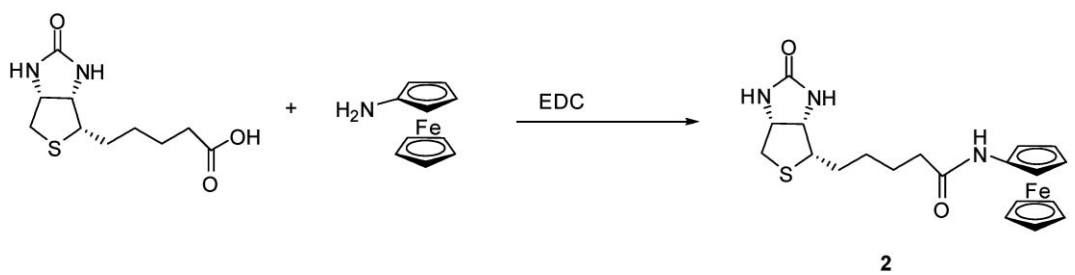
Synthesis of biotinylferrocene (**1a**) and biotinylruthenocene (**1b**) presents interest for a rapidly growing branch of science, bioorganometallic chemistry.[13] It is now well recognized that introduction of ferrocenyl[13,14] and, to a lesser extent, ruthenocenyl[15] groups into biomolecules or biologically active structures may result in novel, interesting features of the conjugates. Furthermore, the redox activity of ferrocenyl conjugates can be exploited in bioanalysis.[13a]

In aqueous solutions, the pyrene conjugate **1c** displayed strong fluorescence, sensitive to the binding of this ligand to avidin (*vide infra*).

For comparison, we also synthesized an analogue of **1a** having an amide bond (**2**, Scheme 2) by coupling of (D)-biotin with aminoferrocene in the presence of EDC (yield 60%).

2.2. Relative binding affinity of 1a–c and 2 for avidin

In the first stage, we qualitatively evaluated the affinity of avidin for **1a–c** and **2** using the HABA assay (HABA = 2-(4′-hydroxyphenylazo)benzoic acid). This assay consists of the displacement of the binding of HABA to avidin by the biotinylated molecules which results in the fading of the characteristic red colour assigned to the HABA-avidin complex (λ_{max} = 500 nm). All the complexes studied readily displaced HABA from avidin, which indicated that their association constants were higher than that of HABA ($K_a = 6 \times 10^6$ M^{-1}).[2] For a semi-quantitative comparison of the affinity of avidin towards **1a–c** and **2** with that of biotin, we carried out a solid-phase competitive enzymatic assay. A microtiter plate coated with avidin was exposed to mixtures of biotin conjugate (or biotin) and biotin-HRP tracer. The amount of the tracer bound to the plate was quantified colorimetrically by addition of enzyme substrates (OPD and H_2O_2). The percentage of bound tracer ($\%B/B_0$) was plotted as a function of log([ligand]) (Fig. 1). Typical sigmoid curves were obtained and non linear



Scheme 2 Coupling of (D)-biotin to aminoferrocene.

Table 1 Values of IC_{50} and %CR for compounds **1a–c** and **2**

Compound	IC_{50}/nM	%CR
Biotin	24 ± 7	100
1a	33 ± 2	73
1b	40 ± 3	60
1c	56 ± 1	43
2	28 ± 2	86

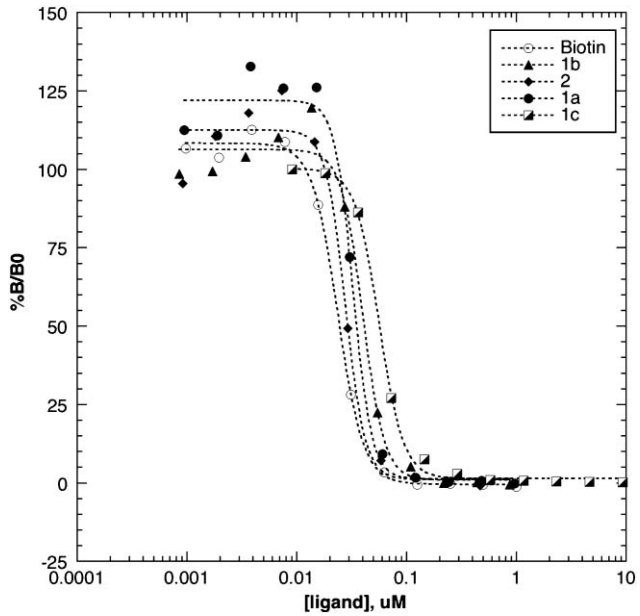


Fig. 1 Plots of B/B_0 as a function of log([ligand]). Dotted lines result from the non linear regression analysis of the data sets using the 4-parameter logistic equation (see Experimental).

regression analysis of the data sets led to the IC_{50} and the percentage of cross-reactivity %CR (Table 1).

All the biotin derivatives displayed a lower binding affinity towards avidin with respect to biotin (%CR are all lower than 100%). Addition of the pyrenyl entity decreased the affinity of biotin by half while addition of the organometallic moieties had less effect on the binding affinity. Therefore, the affinity seems to be related to the bulkiness of the reporter group and the distance between the biotin entity and the reporter group. It is not surprising considering the structure of the biotin-avidin complex. Any substituent attached to the carboxylic end group will be positioned outside the beta-barrel and the biotin binding pocket (see section 2.3.2.4). It is also worth noticing that compounds **2** and **1a** (which only differ by a NH group) displayed comparable affinities. Therefore, the contribution of the amide bond in binding of **2** to avidin is practically negligible.

2.3. Fluorescence properties of 1c

Biotin-fluorophore conjugates are fluorescent molecular probes that may be employed in conjunction with (strept)avidin for various bioanalytical applications (avidin assay, immunoanalysis, histochemistry, gene analysis), provided their association to (strept)avidin does not quench their emission.[16] A large variety of compounds of this class have already been prepared and studied.[17–30] Compound **1c** contains the 1-acylpyrene fluorophore, the fluorescence of which involves (π,π*) and (n,π*) excited states, that are strongly dependent on the polarity of the medium and hydrogen bonding effects.[31] In fact, 1-acylpyrenes have been successfully applied as probes of microenvironments in polymers, biological membranes. micelles and nanorods. The most thoroughly studied compound of this class, pyrene-1-carbaldehyde (1-PCA),[31a–d] exhibits only weak fluorescence in nonpolar solvents but is strongly fluorescent in polar solvents. Furthermore, a bathochromic shift of the emission band in polar solvents is observed. These effects were ascribed to the stabilization of the (π,π*) state with respect to the (n,π*) state due to interaction of the solvent with electron lone pairs on the carbonyl oxygen. The sensitivity of the fluorescence of the 1-acylpyrene fluorophore to the physicochemical properties of its immediate surroundings (environment-sensitive fluorophore[32]) and the high affinity of biotin and biotin derivatives to (strept)avidin (*vide supra*) makes compound **1c** a potential probe for studying biotin-(strept)avidin interaction. Therefore, we decided to study the fluorescence properties of compound **1c** in the absence and presence of avidin.

2.3.1. Electronic absorption and emission spectra of 1c. The electronic absorption spectrum of **1c** in aqueous solution presents two maxima at 293 and 343 nm (Fig. 2). When excitation was carried out at 355 nm, the emission spectrum of **1c** displayed a broad, structureless band centred at 461 nm. This value is close to those exhibited by 1-PCA in aqueous solution, (466 nm),[31a] (469 nm)[31b] and 1-heptanoylpyrene, which may be considered as an analogue of **1c** devoid of the bicyclic moiety (450 nm in methanol, shifted bathochromically by addition of water[31f]). These bands are ascribed to the monomer fluorescence. 1-PCA can also display (in concentrated solutions) fluorescence at ~530 nm ascribed to excimers.[31a,33] On the other hand, a similar emission of 1-heptanoylpyrene was assigned to ground state aggregates.[31e] However, we did not observe any emission of **1c** centred at >500 nm, which reveals that association effects either in the ground or excited state of this compound are negligible and the observed emission band results from excitation of its monomeric form.

2.3.2. Influence of avidin on the fluorescence of 1c.

*2.3.2.1. Titration of **1c** with avidin.* Titration of a solution of **1c** (233 nM) with avidin resulted in a progressive hypsochromic shift of the emission maximum from 461 nm to 425 nm up to the avidin-to-**1c** ratio of 0.43 (Fig. 3).

Conversely, when a solution containing avidin (124 nM) and **1c** (500 nM), which exhibited a maximum of fluorescence at 430 nm (**1c** bound to avidin), was treated with biotin (2.5 μM) a progressive shift of the emission maximum up to 460 nm was observed together with an increase of the intensity of fluorescence at 460 nm (Fig. 4).

This shift and this increase resulted from the displacement of **1c** from its complex with avidin. The final maximum of fluorescence at 460 nm indicated that a 4-fold molar excess of biotin brought about complete displacement of **1c**. The calculated half-life of the **1c**-avidin complex in this experiment was 33 min.

*2.3.2.2. Titration of avidin by **1c**.* Increasing amounts of **1c** (up to 582 nM) were added to a solution of avidin in water (124 nM) and the fluorescence spectrum of the mixture was recorded after each addition (excitation at 355 nm) (Fig. 5). The same experiment was repeated without avidin as a control. In the presence of substoichiometric concentrations of **1c** with respect to

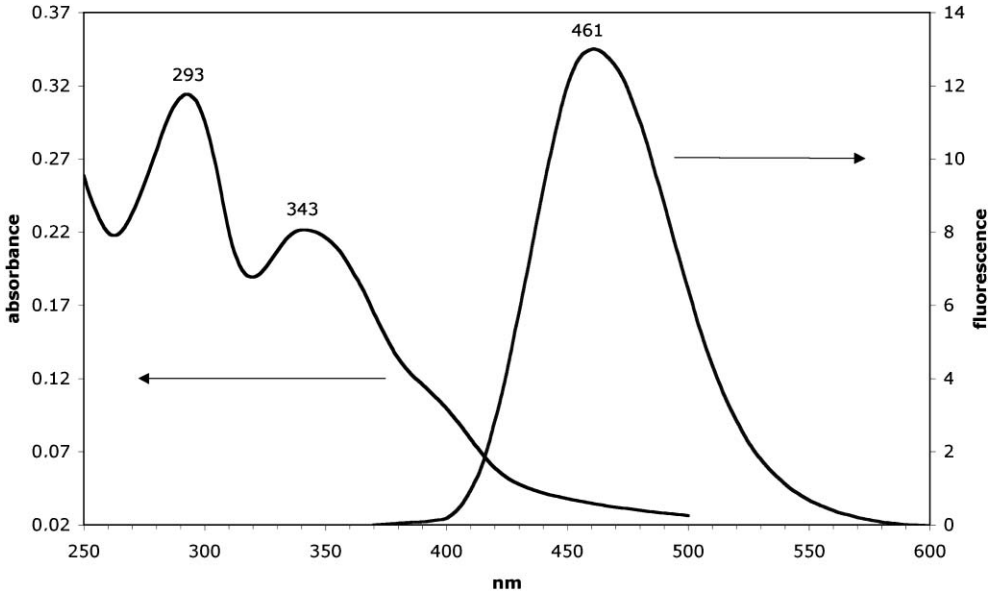


Fig. 2 Absorption and emission (λ_{excit} = 355 nm) spectra of **1c** in aqueous solution.

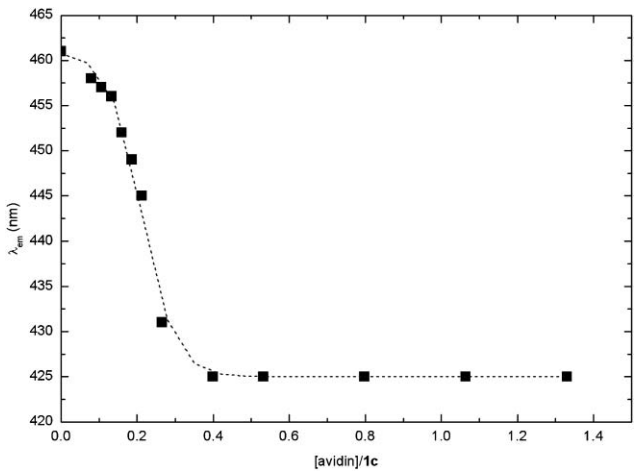


Fig. 3 Titration of **1c** with avidin. Data fitted by nonlinear regression analysis to a sigmoid-type curve.

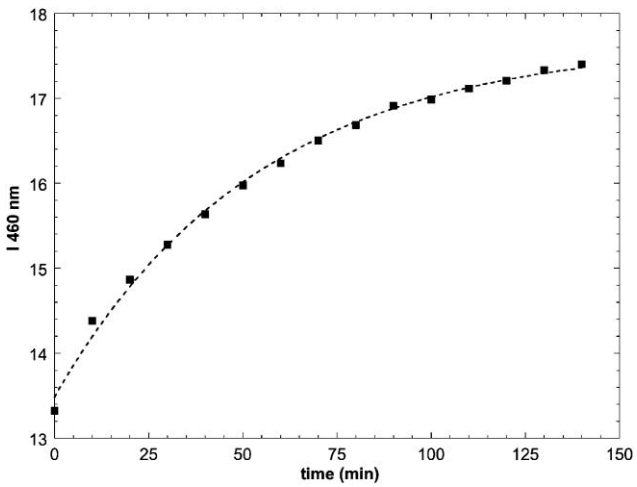


Fig. 4 Plot of the intensity of fluorescence at 460 nm of the **1c**-avidin system *versus* time after addition of excess biotin (for details see text). Data fitted by nonlinear regression analysis to a first order kinetics model.

biotin binding sites, the maximum of emission was at 428 ± 1 nm, indicating that **1c** was complexed to avidin.

At concentrations of **1c** greater than 408 nM, the maximum of emission underwent a red shift, as a result of the presence of unbound **1c** in the medium. The intensity of fluorescence emission at 420 nm was plotted as a function of **1c** in the presence and absence of avidin (Fig. 6, left). Furthermore, the intensity of emission was plotted as a function of the **1c**-to-avidin ratio (Fig. 6, right).

The intensity of emission at 420 nm increased linearly with respect to the concentration of **1c** with a slope equal to 0.039. A sharp inflection point was observed when the concentration of **1c** reached 408 nM, that is a 3.3 : 1 ratio of **1c**. Above this concentration, the intensity of emission increased linearly with a slope of 0.006, close to that measured for free **1c** (0.0087). Binding of **1c** to avidin thus enhanced the emission of fluorescence at 420 nm by a factor of 4.5 as a result of the displacement of the maximum of emission from 460 to 427 nm (while it had practically no effect on the intensity of emission at 460 nm). If **1c** had reacted stoichiometrically with avidin, we would have expected that the breakpoint be reached at a ratio of 4 : 1 as avidin is a tetrameric protein. This may suggest that **1c** is not able to bind to the fourth binding site. Deviation from the ideal 4 : 1 binding stoichiometry was already observed in some cases and explained as a result of steric effects of the ligands.[34–36]

2.3.2.3. Competitive fluorescence assay of biotin. Avidin (124 nM) was mixed with a fixed concentration of **1c** (500 nM) and a variable concentration of biotin (0–1875 nM) in water and the fluorescence emission of the solutions was measured at 420 nm (excitation at 340 nm) and corrected from the fluorescence emission of **1c** at the same concentration. As expected, the fluorescence intensity gradually decreased as the concentration of biotin increased as a result of competitive binding of biotin to avidin. The fraction of bound tracer B/B_0 was plotted as a

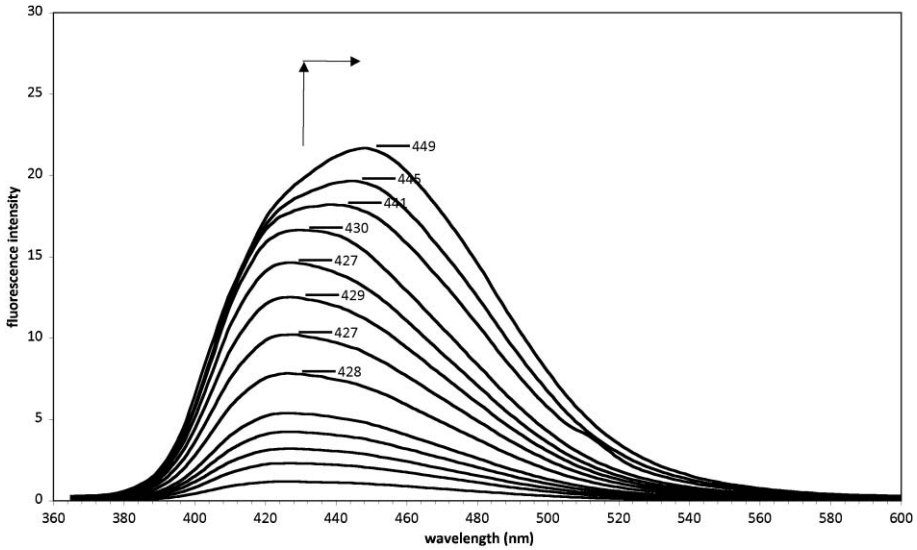


Fig. 5 Fluorescence spectra of mixtures of avidin (124 nM) and **1c** (23–582 nM) in water. Excitation was set at 355 nm.

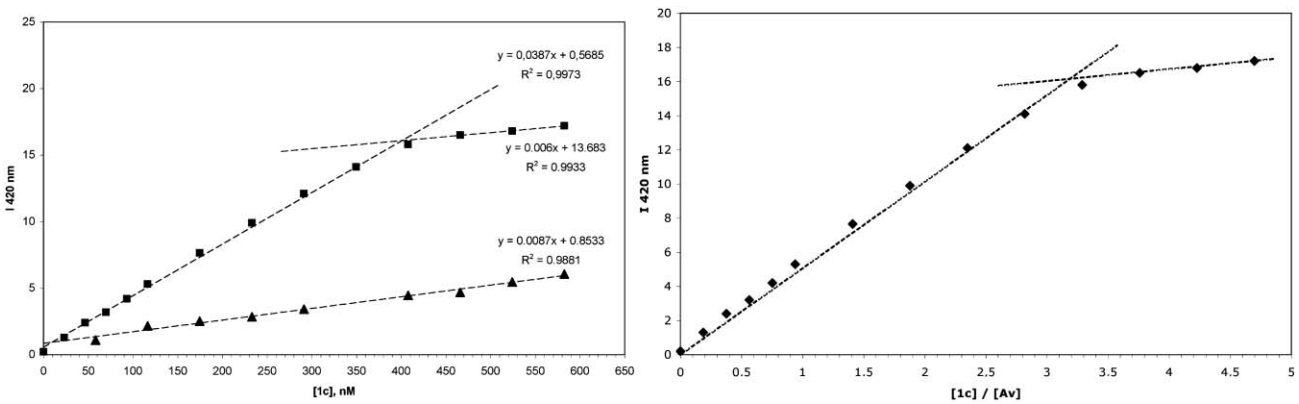


Fig. 6 Left: Intensity of fluorescence at 420 nm of **1c** in the presence (■) and absence (▲) of avidin. Right: Intensity of fluorescence at 420 nm as a function of **1c**-to-avidin ratio (excitation at 355 nm).

function of log([biotin]). A typical sigmoid curve was obtained (Fig. 7) and an IC_{50} of 326 ± 14 nM was determined from the non linear regression analysis of the data set.

The aforementioned results can be interpreted in terms of reversible, specific binding of **1c** to avidin. The resulting hypsochromic shift of the emission band of **1c** suggests that the microenvironment of the avidin binding sites is less polar than the bulk medium, which is in line with the X-ray structure of this protein.[37]

A great variety of biotin-fluorophore conjugates have already been reported in the literature. They include biotin-pyrene derivatives, having the pyrene fluorophore separated from the biotin moiety by PEG-chains of different lengths (PEG_{800} or PEG_{1900}).[17–19] In aqueous solution, the fluorescence spectrum of these molecules relates exclusively to the emission of the pyrene monomer (λ = 380 and 400 nm). Other examples include biotin conjugates with fluorescein[20–23] and other fluorescent dyes,[24] acridine derivatives,[25–27] transition-metal complexes,[28] naphthylamine[29] and BODIPY.[30]

The interaction of the reported fluorophore conjugates of biotin with avidin gave rise to various kinds of behaviour. In most cases, binding to avidin led to the quenching of the fluorophore emission (self-quenching *via* Förster resonance energy transfer mechanism, FRET) [*e.g.* ref. 19, 20] except for biotin-fluorophore conjugates with long spacers.[18] However, in some cases fluorescence enhancement upon binding of the conjugate to avidin was observed [*e.g.* ref. 29, 38] (this is a common behaviour of biotin conjugates with transition-metal complexes[28]). There are also a few examples of conjugates showing a shift of the emission maximum upon formation of complexes with avidin.[39–41]

In comparison with the reported biotin-fluorophore conjugates two features of **1c** are worth noting. Firstly, no self-quenching of fluorescence of **1c** was observed in the presence of avidin. This may be explained by insignificant overlap of emission and absorption spectra of **1c** (Fig. 2) making FRET between molecules of this compound in its complex with avidin inefficient. Secondly, the presence in **1c** of the 1-acylpyrene fluorophore, which is sensitive to the polarity of the microenvironment and hydrogen bonds, brings about a blue shift of the emission band upon formation of its complex with avidin, presumably due to a lower polarity of the protein binding pocket.

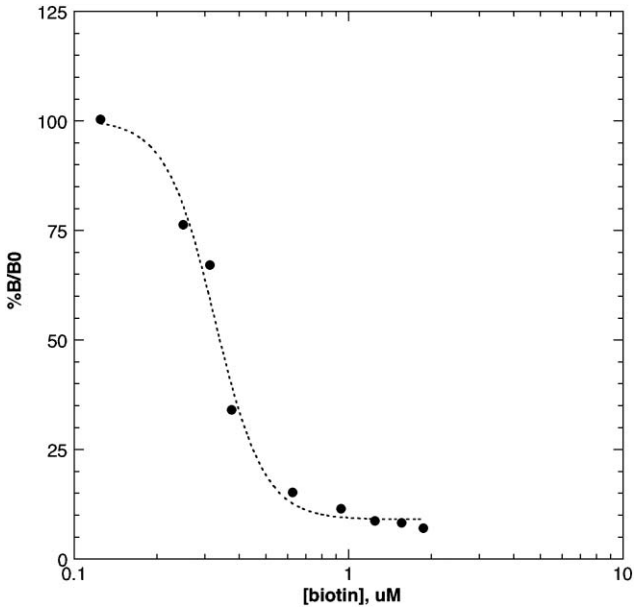


Fig. 7 Competitive fluorescence assay of biotin. Dotted line results from the non linear regression analysis of the data set using the 4-parameter logistic equation (see Experimental).

*2.3.2.4. Förster resonance energy transfer (FRET) in the avidin-**1c** complex.* Fluorescent probes can also be excited indirectly *via* Förster resonance energy transfer (FRET) from the fluorophore present in the biomolecule binding the probe.[42] In recent years FRET has proved to be a powerful technique in bioanalysis, especially in the detection of molecular binding events and changes in protein conformation because of its intrinsic sensitivity (proportional to R^{-6}, where R is the distance between the fluorophore present in the biomolecule (D) and that present in the probe (A)). FRET occurs when the emission spectrum of D overlaps the absorption spectrum of A and where $R < 10$ nm (100 Å).

All the proteins contain aromatic amino acids such as phenylalanine, tyrosine and tryptophan, which are potential donors. Fluorescence arising from excitation of tryptophan residues is quite informative owing to its high sensitivity to their local environment. When excited at 280 nm, avidin displayed a broad emission band at 342 nm (Fig. 8). This band overlaps substantially with the emission band of **1c** (Fig. 8), revealing that FRET between these molecules is possible. When excess biotin was added to a solution of avidin, a blue shift of the emission band was observed to 333 nm together with a quenching of emission (30%) (Fig. 8). These effects are caused by conformational changes of avidin (movement of the tryptophan residues to the nonpolar, internal environment) resulting from the binding of biotin.[43]

When excess **1c** was added to avidin the intrinsic fluorescence of the protein was almost fully quenched (95%) while the emission of the ligand occurred at 430 nm instead of 460 nm for the free ligand as previously observed. Addition of excess biotin almost fully restored the intrinsic fluorescence of the protein (332 nm) by preventing the binding of **1c** to avidin, while **1c** emitted at 460 nm. The latter fluorescence corresponds to the direct excitation of **1c**, which absorbs at 280 nm (Fig. 9). Obviously, the emission of the avidin-**1c** complex at 430 nm can partly result from the direct excitation of the ligand, but the efficient quenching of the protein fluorescence suggests that FRET is the major pathway.

A similar situation has recently been described in the literature involving a naphthylamine derivative of biotin and its interaction with streptavidin.[29] A FRET process was seen to occur between streptavidin acting as an intrinsic donor and the naphthylamine derivative acting as the acceptor, producing simultaneous quenching of the fluorescence of streptavidin and enhancement of the fluorescence of the probe.

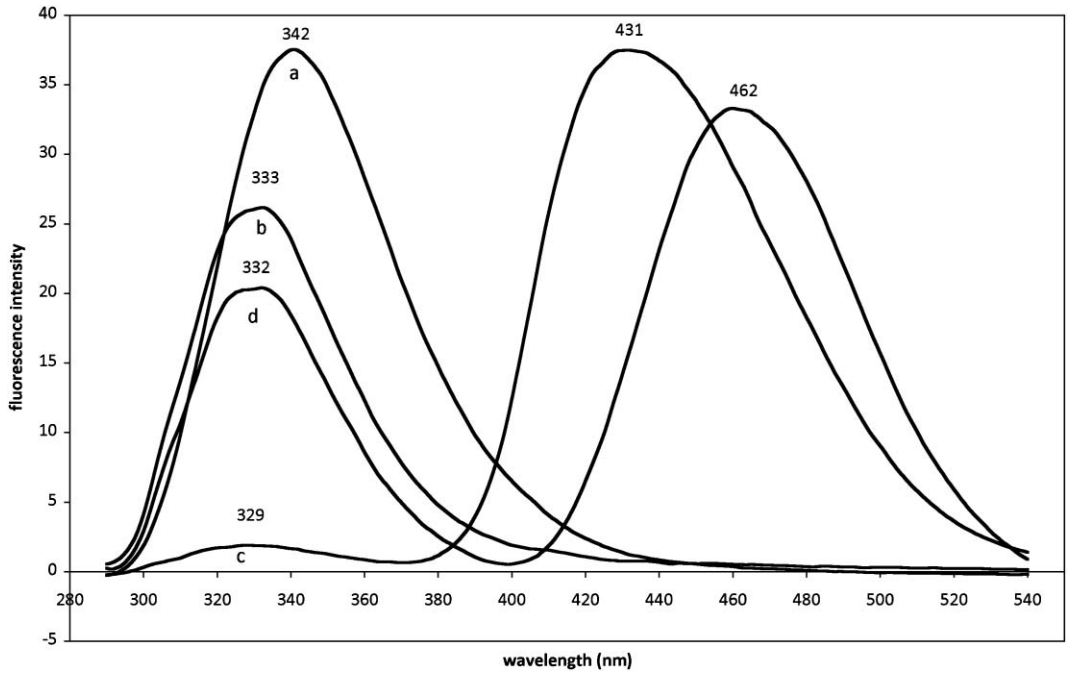


Fig. 8 Emission spectra of avidin (a) and avidin-biotin (b), avidin-**1c** (c) and avidin-biotin-**1c** (d) systems (excitation at 280 nm). For details see text.

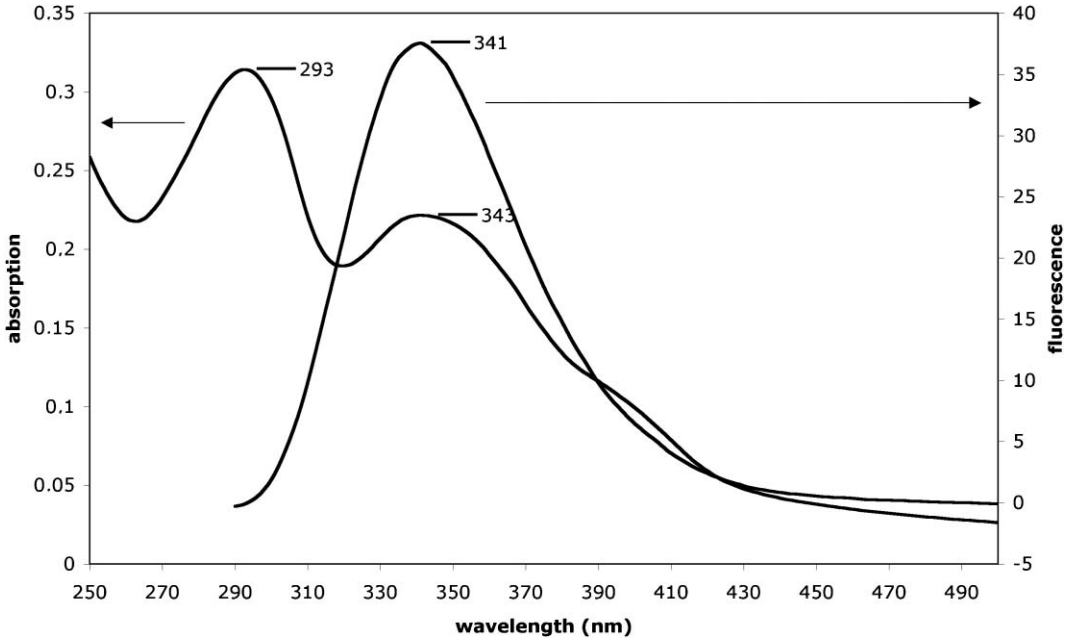


Fig. 9 Absorption spectrum of **1c** and emission spectrum of avidin (excitation at 280 nm).

2.3.2.5. Docking of 1c into avidin monomer. **1c** was manually constructed from the 3D structure of biotin extracted from the avidin-biotin complex 3D structure (PDB file 1avd) and its structure minimized by molecular mechanics calculation (Chem 3D). Docking of **1c** into the avidin monomer scaffold was carried out with Molegro molecular docker (Fig. 10).

When compared to the 3D structure of the avidin-biotin complex, it was observed that the hydrogen bonds between the protein scaffold and some of the H-bond acceptors/donors of the biotin-pyrene ligand were entirely conserved, especially the H-bond between the carbonyl group linking biotin to pyrene and Ser73 and the one between the carboxylic group of (D)-biotin and Ser73. The pyrene entity is pointing out of the barrel but closely interacts with amino acids S101 and S102. In the avidin-**1c** complex the distances between the pyrene fluorophore and the four tryptophan residues in the avidin monomer are all within the Förster distance, with three of the donors (W70, W97 and W110) being at a distance less than 10 Å from the fluorophore, making FRET very efficient.

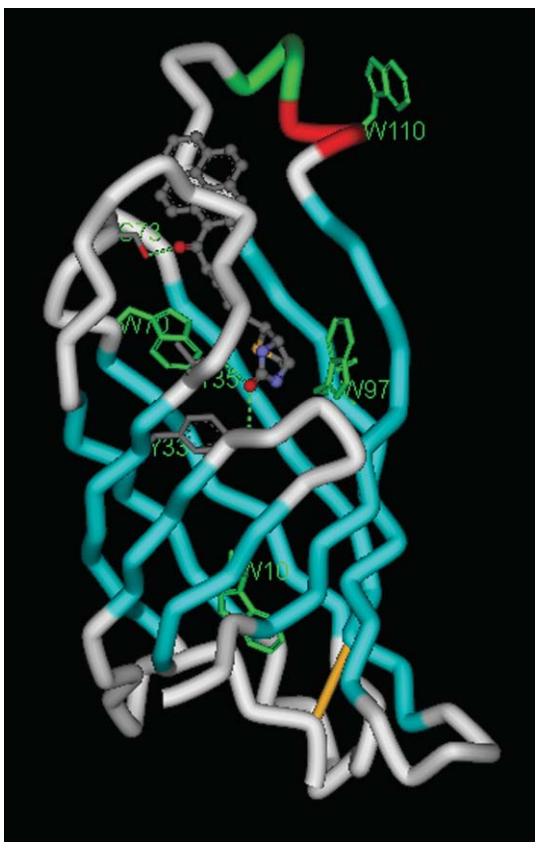


Fig. 10 Docking of **1c** within the avidin monomer.

3. Conclusions

To the best of our knowledge the reaction shown in Scheme 1 constitutes the first example of the use of biotin as the acylating agent in a Friedel–Crafts reaction. The aromatic biotinylated ketones formed in this reaction may find application in various biochemical procedures. This new coupling mode preserves a high affinity of the conjugates for avidin and the absence of the amide bond should make them resistant to the enzyme biotinidase,[12] which catalyses the cleavage of C–N bonds in biotin amides. They also contain a reactive ketonic C═O group which offers the possibility of various structural transformations. The reported reaction provides an alternative for biotinylation methods based on carboxyl-amine coupling.

In the context of our work it is worth noting that recently a terminal alkyne derived from biotin was synthesized and conjugated with a phenylalanine derivative (containing iodide in the phenyl ring) through C–C bond-forming Sonogashira coupling.[7b] The conjugate obtained in this way, although lacking

the carbonyl group at the end of the valeric acid side chain, exhibited high affinity to avidin, with the ability to displace HABA in its complex with avidin. This result, along with that presented in this work, demonstrates that C–C bond forming reactions should also be considered as an alternative method of construction of biotinylated molecules having a high affinity to (strept)avidin.

Experimental Section

General remarks

All reactions were carried out under argon. All reagents used in this work are commercially available (Sigma-Aldrich) and were used without further purification. Solvents were dried over appropriate drying agents and distilled before use. Flash chromatography was performed using Analogix IntelliFlash 310 system (Varian Inc.) equipped with SuperFlash, Si 50 columns (SF10-8g). The NMR spectra were run on Varian Gemini 200 BB (200 MHz for ^{1}H), Bruker Advance 250 (250 MHz for ^{1}H) and Bruker Avance II Plus 700 (700 MHz for ^{1}H) spectrometers. The IR spectra were recorded on a Bruker Tensor (Miracle ATR accessory, Ge crystal) spectrometer in ATR mode. Mass spectra were measured on a Finnigan MAT 95 spectrometer. Elemental analyses were performed by the Analytical Services of the Center of Molecular and Macromolecular Studies of the Polish Academy of the Sciences (Łódź). Aminoferrocene was prepared analogously as 1′,2′,3′,4′,5′,-pentaphenylaminoferrocene.[44] Hen egg white avidin was purchased from Interchim. A 10 mg solid mL^{-1} stock solution was prepared in water and kept at 4 °C. The actual concentration of avidin was determined by the colorimetric HABA assay[45] and was found to be equal to 126 μM. Stock solution of (D)-biotin (2.5 mM) and **1c** (2.33 mM) were prepared in water and DMSO, respectively. HRP-biotin conjugate was purchased from Sigma. UV-visible spectra were recorded on a uv/mc2 spectrophotometer (Safas). Fluorescence spectra were recorded on an F-6200 spectrofluorimeter (JASCO) at 20 ± 0.1 °C. The excitation and emission slit widths were set at 5 nm. The scan speed was 125 nm min^{-1} with a data pitch of 1 nm. Spectra of aqueous solutions of **1c** (containing 1% DMSO for solubility reasons) were recorded in a 1 cm-pathlength quartz cell between 360 and 600 nm by setting the excitation wavelength at 355 nm. For the FRET experiments, fluorescence spectra of proteins were recorded between 260 and 540 nm by setting the excitation wavelength at 280 nm.

General procedure for the synthesis of biotinylarenes 1a–c

To a suspension of biotin (1 mmol) in dichloromethane (5 mL), trifluoroacetic anhydride (210 mg, 150 μL, 1 mmol) was added and the mixture was stirred until biotin was completely dissolved. Then the arene (1 mmol) and triflic acid (150 mg, 84 μL, 1 mmol) were added and the mixture was stirred at r.t. for the time indicated below. After quenching with water the products were isolated by extraction with dichloromethane and flash chromatography (0–5% methanol in dichloromethane within 5 min for elution).

Biotinylferrocene (1a)

Reaction time 2 h. Yield 55%. ^{1}H NMR (400 MHz, DMSO-d_6): 6.53 (bs, 1H, NH); 6.42 (bs, 1H, NH); 4,86 (t, J = 1.8 Hz, 2H, Cp); 4.62 (t, J = 1.8 Hz, 2H, Cp); 4.38 (dd, J = 7.6, 5.2 Hz; 1H, CH); 4.29 (s, 5H, Cp); 4.24–4.20 (m, 1H), 3.22–3.17 (m, 1H); 2.90 (dd, J = 12.4, 4.8 Hz, 1H); 2.79 (t, J = 7.2 Hz, 2H); 2.65 (d, J = 12.4 Hz, 1H); 1.73–1.43 (m, 6H, CH_2). ^{13}C NMR (100 MHz, DMSO-d_6): 203.37 (CO-amide); 162.69 (CO); 79.05(Cp-ipso); 71.96 (Cp); 69.49 (Cp′); 69.03 (Cp); 61.05 (CH); 59.15 (CH); 55.47 (CH); 39.86 (CH_2); 38.57 (CH_2); 28.39 (CH_2); 28.23 (CH_2); 24.06 (CH_2). IR (cm^{-1}): 3227, 2926, 1707, 1666. MS: 435.1 (M+Na)$^+$. Elemental analysis: Calcd. for $C_{20}H_{24}FeN_2O_2S{\cdot}2.5H_2O$ C-52.52; H-6.39; N-6.12; S-7.01 Found: C-52.91; H-5.59; N-6.18; S-7.05.

Biotinylruthenocene (1b)

Reaction time 4 h. Yield 53%. ^{1}H NMR (400 MHz, DMSO-d_6): 6.47 (bs, 1H, NH); 6.37 (bs, 1H, NH); 5.11 (t, J = 1.6 Hz, 2H, Cp); 4.83 (t, J = 1.6 Hz, 2H, Cp); 4.63 (s, 5H, Cp); 4.33–4.30 (m, 1H); 4.16–4.14 (m, 1H); 3.14–3.01 (m, 1H); 2,83 (dd, J = 12.4, 5.2 Hz, 1H); 2.61–2.57 (m, 3H); 1.61–1.32 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): 201.43 (CO-amide); 162.70 (CO); 83.86 (Cp-ipso); 73.43 (Cp); 71.79 (Cp′); 70.56 (Cp); 61.05 (CH); 59.15 (CH); 55.46 (CH); 39.85 (CH_2); 37.89 (CH_2); 28.28 (CH_2); 28.16 (CH_2); 24.26 (CH_2). IR (cm^{-1}): 3233, 2925, 1713, 1672. MS: 481.1 (M+Na)$^+$. Elemental analysis: $C_{20}H_{24}RuN_2O_2S{\cdot}0.5H_2O$ C-51.49; H-5.40; N-6.00; S-6.87; Found: C-51.41; H-5.42; N-5.81; S-6.23.

1-Biotinylpyrene (1c)

Reaction time 4 h. Yield 47%. ^{1}H NMR (600 MHz, DMSO-d_6): 8.75 (d, J = 9.3 Hz, 1H, CH); 8.55 (d, J = 8.0 Hz, 1H, CH); 8.41–8.38 (m, 3H, 3xCH); 8.34 (d, J = 9.4 Hz, 1H, CH); 8.33 (d, J = 8.9 Hz, 1H, CH); 8.27 (d, J = 8.9 Hz, 1H, CH); 8.16 (t, J = 7.6 Hz, 1H, CH); 6,46 (s, 1H, NH); 6.37 (s, 1H, NH); 4.31 (dd, J = 7.56, 5.16 Hz, 1H, CH); 4.17–4.14 (m, 1H, CH); 3.30–3.26 (m, 2H, CH_2); 3.15–3.12 (m, 1H, CH); 2.83 (dd, J = 12.4, 5.1 Hz, 1H, CH_2); 2.59 (d, J = 12.4 Hz, 1H, CH_2); 1.78 (m, 2H, CH_2); 1.74–1.76 (m, 1H, CH_2); 1.56–1.54 (m, 1H, CH_2); 1.50–1.47 (m, 2H, CH_2).^{13}C NMR (150 MHz, DMSO-d_6): 204.82 (C); 162.68 (C); 132.96 (C); 132.71 (C); 130.63 (C); 129.94 (C); 129.27 (CH); 129.22 (CH); 128.13 (C); 127.17 (CH); 126.74 (CH); 126.51 (CH); 126.40 (CH); 125.97 (CH); 124.44 (CH); 124.36 (CH); 124.00 (C); 123.50 (C); 61.04 (CH); 59.15 (CH); 55.40 (CH); 41.73 (CH_2); 40.01 (CH_2); 39.85 (CH_2); 28.20 (CH_2); 24.32 (CH_2). IR cm^{-1}: 3231, 2926, 1703, 1680. MS: 451.1 (M+Na)$^+$. Elemental analysis: Calcd for $C_{26}H_{24}N_2O_2S{\cdot}0.5H_2O$ C- 71.37; H-5.76; N-6.40; S-7.33. Found: C-71.78; H-5.57; N-6.49; S-7.25.

Synthesis of biotinamidoferrocene (2)

A solution of aminoferrocene (201 mg, 1 mmol), biotin (210 mg, 0.87 mmol), and EDC (660 mg, 3.45 mmol) in 1 : 1 water-ethanol (30 mL) was stirred at r.t. for 3 h. Then the solvents were evaporated and a crude product was purified by flash chromatography using dichloromethane-methanol gradient (0–10% MeOH in dichloromethane within 5 min) for elution. Yield: 242 mg (60%). ^{1}H NMR (400 MHz, CD_3OD) δ 4.69–4.56 (m, 2H, Cp), 4.50 (dd, *J* = 7.8, 4.6 Hz, 1H, CH), 4.32 (dd, *J* = 7.8, 4.4 Hz, 1H, CH), 4.15 (s, 5H, Cp), 4.00 (t, *J* = 1.9 Hz, 2H, CH_2), 3.29–3.16 (m, 1H, CH_2), 2.94 (dd, *J* = 12.7, 5.0 Hz, 1H, CH_2), 2.72 (d, *J* = 12.7 Hz, 1H, CH_2), 2.29 (t, *J* = 7.3 Hz, 2H, CH_2), 1.87–1.59 (m, 4H, CH_2), 1.52 (dd, *J* = 15.2, 7.5 Hz, 2H, CH_2).

^{13}C NMR (100 MHz, CD_3OD): 174.33 (CO); 166.13 (CO); 95.84 (Cp-ipso); 70.16 (CH); 65.46 (CH); 65.44 (CH); 63.43 (CH); 62.58 (CH); 62.54 (CH); 61.64 (CH); 57.08 (CH); 41.12 (CH_2); 37.38 (CH_2); 29.89 (CH_2); 29.62 (CH_2); 27.00 (CH_2).

IR (cm^{-1}): 3227, 2926, 1693, 1657. MS: 450.1 $(M+Na)^+$ Elemental analysis: Calculated for $C_{20}H_{25}FeN_3O_2S{\cdot}0.5H_2O$: C-55.05; H-6.01; N-9.63; S-7.35; Found: C-54.65; H-6.12; N-9.28; S-6.91.

Competitive binding assay for 1a–c and 2

A polystyrene 96-well microtiter plate (Greiner) was treated with a solution of avidin (10 μg mL^{-1}, 100 μL $well^{-1}$) in carbonate buffer pH 9.5 overnight at 4 °C. The plate was blocked by addition of PBS containing 1% BSA (w/v) (100 μL $well^{-1}$) for 30 min at r.t. and washed 3 times with PBS containing 0.05% Tween 20 (150 μL $well^{-1}$). Standard solutions of biotin, **1a–c** or **2** (0–2 μM) were applied to 5 series of Micronic tubes (0.2 mL $tube^{-1}$) and biotin-HRP solution (40 ng mL^{-1}; 0.2 mL $tube^{-1}$) was added to each tube. A 150 μL amount of each solution was added in duplicate to the plate that was incubated 40 min at r.t. in the dark. The plate was washed 3 times with PBS-0.05% Tween 20 and an OPD solution in citrate-phosphate buffer pH 5 and 0.16% H_2O_2 v/v (0.7 mg mL^{-1}; 100 μL $well^{-1}$) was added to the wells. After sufficient colour development, the enzymatic reaction was stopped by H_2SO_4 (2.5 M; 50 μL $well^{-1}$), and the absorbance at 485 and 410 nm was read with a microtiter plate reader (BMG Labtech). Data sets were fitted by non linear regression analysis (Kaleidagraph) applying the 4-parameter logistic eqn (1).

$$\%B/B_0 = d + \frac{a-d}{1+([\text{ligand}]/c)^b} \quad (1)$$

where a and d are the upper and lower asymptotes (typically equal to 100 and 0), respectively; c is the value of [ligand] at the inflexion point (*i.e.* IC_{50}) and b is related to the slope at the centre of the sigmoid. The percentage of cross-reactivity %CR or relative binding affinity is defined by eqn (2):

$$\%CR = \frac{IC_{50}(\text{biotin})}{IC_{50}(\text{ligand})} \times 100 \quad (2)$$

Titration of 1c with avidin

To a solution of **1c** (233 nM; 2.02 mL) were added aliquots (1–10 μL) of avidin solution (12.4 μM). Mixtures were magnetically stirred for 5 s and the fluorescence spectrum recorded 2 min after each addition.

Displacement of 1c from the avidin-1c complex by (D)-biotin

An aqueous solution of avidin (124 nM) and **1c** (500 nM) was incubated for 15 min and the fluorescence emission spectrum recorded. An aliquot (2 μL) of (D)-biotin stock solution (25 μM final concentration) was added to the solution and after brief stirring the fluorescence emission spectrum was recorded every 10 min for 3 h.

Titration of avidin by 1c

To a solution of avidin (124 nM, 2 mL) were added aliquots (2–5 μL) of **1c** solution (23 μM). Mixtures were magnetically stirred for 5 s and the fluorescence spectrum recorded 2 min after each addition.

Fluorescence competitive assay of biotin

Solutions (2 mL) containing avidin (124 nM), **1c** (500 nM) and biotin (0–1875 nM) were incubated for 1 h at r.t. The fluorescence intensity of the solutions at 420 nm F (λ_{excit} = 340 nm) was measured. The fluorescence intensity of a solution of **1c** at the same concentration F_c was also measured. $(F–F_c)/(F_0–F_c)$ (F_0 is the fluorescence intensity measured for [biotin] = 0) was plotted as a function of log([biotin]). Data were fitted to the 4-parameter logistic eqn (1) as above.

Docking experiment

Compound **1c** was built using Chem3D Ltd. 9.0 (Cambridgesoft) on the basis of the X-ray structure of (D)-biotin extracted from the avidin-biotin 3D structure (PDB file 1avd) and its structure minimized by applying the molecular mechanics algorithm (MM2). Docking of **1c** into the avidin monomer set as rigid was performed with Molegro virtual docker (Molegro).[46] The binding site was automatically detected by the docking software. Ten runs were performed and five poses returned. All the other docking parameters were set as default. The pose for which the biotin entity of **1c** binds to avidin in a similar way as in the original biotin-avidin complex was exported as a file and examined with Discovery Studio Visualizer 2.0 (Accelrys).

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