Bioorganometallic Chemistry of Ferrocene

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Contents

1. Introduction

In 1951, a new compound containing iron and two cyclopentadienide ligands was reported.[1,2] Although even the first reports noted its high and unexpected stability, the correct structure was only soon afterward suggested independently by Wilkinson[3] and Fischer.[4] Owing to the resemblance of its reactivity to that of benzene, the name *ferrocene* (**1**) was coined for the new compound by Woodward.[5] The term "sandwich compound" for this compound is today universally accepted for a much wider class of compounds. The discovery of ferrocene and elucidation of its remarkable structure is arguably the starting point for modern organometallic chemistry. In recent years, *bioorganometallic chemistry* has developed as a rapidly growing and maturing

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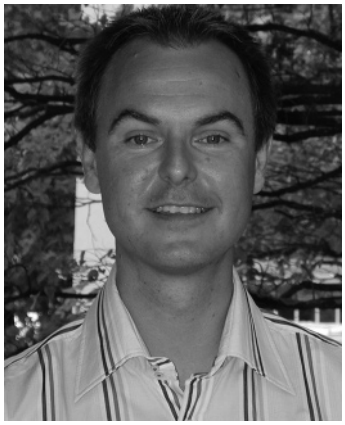


Dave R. van Staveren was born in Rotterdam, The Netherlands, in 1976. He studied chemistry at Leiden University from 1994 to 1999 and conducted his diploma research in the group of Professor Jan Reedijk. Subsequently, he moved to the Max-Planck-Institut für Strahlenchemie (nowadays known as the Max-Planck-Institute for Bioinorganic Chemistry) in Mülheim an der Ruhr, Germany, to perform Ph.D. research in the group of Professor Karl Wieghardt, under the supervision of Nils Metzler-Nolte. He was awarded a Ph.D. degree from the University of Bochum in 2001. He then switched to the Max-Planck-Institut for Kohlenforschung, the other Max-Planck-Institute in Mülheim an der Ruhr, to work as a postdoctoral fellow with Professor Alois Fürstner in the field of alkyne metathesis from 2001 through most of 2002. After spending half a year at the University of Heidelberg, Germany, as a teaching assistant in the pharmacy department, he moved to Switzerland to work on an industrially financed project involving the radiolabeling of Vitamin B12 with ^{99m}Tc. The first part of this project was completed at the University of Zurich under supervision by Professor Roger Alberto (2003–2004), whereas the second part is currently in progress at the Paul Scherrer Institut in Villigen in the group of Professor P. August Schubiger.

Nils Metzler-Nolte was born in Hamburg, Germany, in 1967. After studying chemistry at the Universities of Hamburg and Freiburg, he moved to the Ludwig-Maximilians-University of Munich in 1989, where he obtained his diploma in chemistry in 1991 and his Ph.D. in 1994, both on organoboron chemistry under the supervision of Professor H. Nöth. He did his postdoc with M. L. H. Green in Oxford and then moved to the Max-Planck-Institute für Strahlenchemie (nowadays know as the Max-Planck-Institute for Bioinorganic Chemistry), where he started his independent research on bioorganometallic chemistry and worked as head of the NMR service group. He obtained his Habilitation from the University of Bochum in May 2000. Soon after, he was appointed full professor for Bioinorganic Chemistry at the University of Heidelberg's Faculty of Pharmacy. He is nowadays located at this University's Institute for Pharmacy and Molecular Biotechnology, which is part of the Faculty of Biosciences. His research interest is in bioorganometallic chemistry and generally functional bioconjugates with transition metals, such as metal–peptide and metal–PNA conjugates, including aspects of medicinal inorganic chemistry. His work has been recognized by several awards, and he was cochairman of the Second International Symposium on Bioorganometallic Chemistry in July 2004 in Zürich, Switzerland.

area which links classical organometallic chemistry to biology, medicine, and molecular biotechnology.[6−8]

The stability of the ferrocenyl group in aqueous, aerobic media, the accessibility of a large variety of derivatives, and its favorable electrochemical properties have made ferrocene and its derivatives very popular molecules for biological applications and for conjugation with biomolecules. This review covers bioconjugates of ferrocene with amino acids and peptides (section 2), proteins (section 3), DNA, RNA, and PNA (section 4), carbohydrates (section 5), and hormones and others (section 6). We have mostly limited our discussion to conjugates in which ferrocene is covalently bonded to the biomolecule, and their application. Applications in which free ferrocene serves a biological purpose, for example, as an electron mediator between enzymes and an electrode, are not considered. Also, we do not discuss the physiological properties of ferrocene or its derivatives. Medicinal applications of ferrocene derivatives are also not covered comprehensively in this review. Ferrocene itself exhibits interesting properties as an anti-aenemic or cytotoxic agent.[9,10] Neuse and co-workers found greatly enhanced activity of cytotoxic metal compounds including ferrocene when these were bound to polymers as prodrugs.[11,12] Conjugates of ferrocene with well-known drugs were reported, for example, with antibiotics such as penicillins and cephalosporins.[13−23] In addition, structural variations of established drugs with the ferrocenyl moiety were reported, such as ferrocenyl aspirin,[24−26] the anti-malarial drugs chloroquine (termed ferroquine), quinine, mefloquine, and artemisinin,[27−39] and the anti-cancer drug tamixofen to give ferrocifen.[40−42]

This review covers the literature from 1957, when Schlögl reported the first ferrocenyl amino acids,[43] through the beginning of 2004. There is no previous review that we are aware of which covers the topic of this article in a concise manner. A book by Togni on ferrocene chemistry does not touch biological aspects.[44] Bergs, Severin, and Beck have published a fairly comprehensive article on bioorganometallic derivatives of amino acids and peptides.[45] Ryabov has reviewed the interaction of organometallic compounds with enzymes and proteins.[46] Very recently, Salmain and Jaouen published a review on the covalent labeling of proteins with organometallic complexes.[47] In a series of annual surveys on ferrocene in the *Journal of Organometallic Chemistry*,[48−65] one chapter was usually devoted to biological applications of this particular organometallic compound. Two older reviews on the biological chemistry of metallocenes have appeared.[66,67] More specialized reviews cover related aspects of ferrocene chemistry, such as, for instance, applications of the compound in glucose biosensors[68] and bioelectronics.[69,70] Very recently, an excellent Russian review on ferrocene-containing nucleic acids was published.[71]

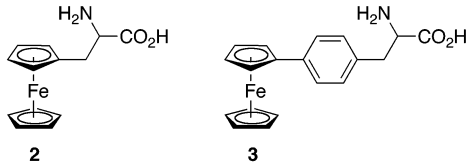
2. Conjugates of Ferrocene with Amino Acids and Peptides

2.1. Unnatural Amino Acids Containing a Ferrocenyl Side Chain

2.1.1. Ferrocenylalanine and Conjugates Thereof

The first two ferrocene amino acid analogues were reported by Schlögl as early 1957, only six years after the initial reports on the synthesis of ferrocene.[1,2] These analogues constitute (1) DL-ferrocenylalanine (DL-Fer, **2**)[43,72,73] and (2) DL-ferrocenylphenylalanine (**3**)[43] (Scheme 1). The latter has never been used after

Scheme 1. Molecular Structure of Ferrocenylalanine (2) and Ferrocenylphenylalanine (3)



its initial report, whereas ferrocenylalanine has been subject of extensive research. A few decades later, several other reports appeared on the (stereoselective) synthesis of this amino acid.[74−77] Two strategies have been adapted to obtain ferrocenylalanine in enantiomerically pure form: either via resolution of the racemic mixture or via stereospecific synthesis. Resolving the racemic mixture can be achieved by (1) fractional crystallization of the diastereomeric brucine salt[76] or (2) kinetic resolution by stereoselective deacylation of the acylated racemate using the enzyme acylase.[77] Ferrocenylalanine can be stereospecifically synthesized with up to 94% ee via asymmetric hydrogenation of the corresponding *Z*-configured dehydro acyl derivatives **4** (Scheme 2), to give *N*-acetyl-protected L-**2** (**5**) followed by deprotection.[75] Another elegant method for obtaining enantiomerically pure Boc-protected ferrocenylalanine **6** consists of a Pd(0)-catalyzed cross-coupling between 1-iodoferrocene (**7**) and the serine-derived organozinc reagent **8** (Scheme 2).[74]

A variety of peptides that contain ferrocenylalanine as an unnatural amino acid have been synthesized. This group of compounds can be divided into two classes, depending on their degree of relevance to biologically occurring peptides. Biologically irrelevant peptides that have been prepared constitute cyclo(D-ferrocenylalanyl-L-prolyl), cyclo(L-ferrocenylalanyl-L-prolyl),[76] and polypeptides containing repeating L-Fer-[Glu(OBzl)]$_4$ or L-Fer$_2$-[Glu(OBzl)]$_4$ units.[77] The other class comprises modified biogenic peptides in which ferrocenylalanine has been specifically substituted for a phenylalanine residue. Compared to Phe, the cylindrically shaped Fer is much more bulky and lipophilic. Owing to these differences, results from binding assays for the modified peptide in comparison to those for the natural peptide could reveal important information about substrate−receptor interactions.

The first biogenic peptide that was modified with Fer is the pentapeptide [Leu^5]-enkephalin, which has primary structure H-Tyr-Gly-Gly-Phe-Leu-OH (**9**, Scheme 3). [Leu^5]-Enkephalin was first isolated as a

Scheme 3. Amino Acid Sequence of [Leu^5]-Enkephalin (9) and [Fer^4, Leu^5]-Enkephalin (10)

H-Tyr-Gly-Gly-Phe-Leu-OH

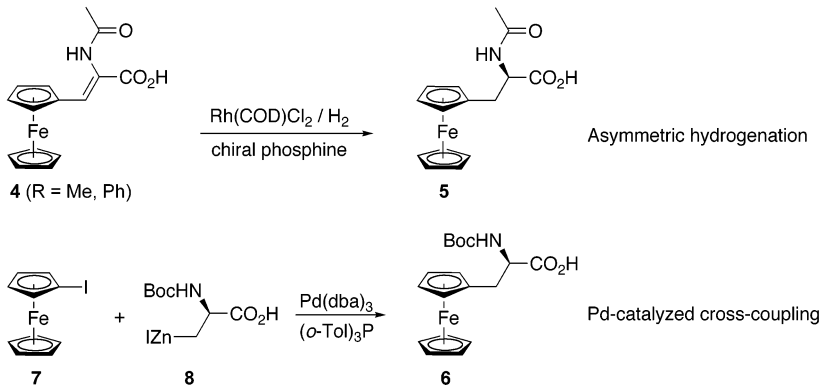
[Leu^5]-Enkephalin (**9**)

H-Tyr-Gly-Gly-Fer-Leu-OH

[Fer^4, Leu^5]-Enkephalin (**10**)

mixture with its position 5 methionine analogue ([Met^5]-enkephalin) by Hughes et al. from pig brain in 1975.[78] The enkephalins are neuropeptides with an action similar to that of morphine, exhibiting high affinity for the opioid receptor. These pentapeptides were detected in various human tissues, such as the human brain, in human spinal fluid, and in blood plasma.[79] Three reports on the synthesis of [Fer^4, Leu^5]-enkephalin, in which the phenylalanine residue in position 4 has been replaced by ferrocenylalanine (**10**, Scheme 3), appeared more or less simultaneously in the literature.[80−82] The compounds were synthesized on a solid support, by employing classical Merrifield solid phase peptide synthesis techniques. Diastereomerically pure [D-Fer^4]- and [L-Fer^4]-enkephalins were obtained either by HPLC purification of the racemic [DL-Fer^4, Leu^5]-enkephalin mixture[82] or by performing the solid-phase peptide synthesis with enantiomerically pure L-Fer and D-Fer.[81] Either diastereomer displays a much reduced affinity for the enkephalin receptor

Scheme 2. Two Methods for Obtaining Enantiomerically Pure Ferrocenylalanine (2)



compared to [Leu^5]-enkephalin,[80,82] although both are significantly more potent than many other posititon 4 analogues.[83] Interestingly, the [D-Fer^4]-diastereomer was significantly more potent than the L-analogue.[80,82]

Substance P (SP, **11**) is an undecapeptide, belonging to the class of tachykinins, with two consecutive Phe residues in positions 7 and 8 (Scheme 4). The

Scheme 4. Primary Structure for Substance P (SP, 11) and Ferrocenylalanine Derivatives 12−14 Thereof

H-Arg-Pro-Lys-Pro-Gln-Gln-X-Y-Gly-Leu-Met-NH_2

X = Phe,	Y = Phe	: SP	(**11**)
X = Fer,	Y = Phe	: [Fer^7]-SP	(**12**)
X = Phe,	Y = Fer	: [Fer^8]-SP	(**13**)
X = Fer,	Y = Ile	: [Fer^7, Ile^8]-SP	(**14**)

peptide was first discovered by von Euler and Gaddum in 1931,[84] but it took another four decades before its amino acid sequence was determined by Chang et al.[85,86] SP is involved in several important physiological processes, such as contraction of the smooth muscles, pain transmission, and activation of the immune system.[87] Tartar and co-workers synthesized the following three Fer-modified SP-derivatives: [DL-Fer^7]-SP (**12**), [DL-Fer^8]-SP (**13**), and [DL-Fer^7, Ile^8]-SP (**14**).[88] As discussed above for enkephalin, one or both Phe residues were replaced by Fer. Racemic Fer was used for the syntheses, resulting in diastereomeric mixtures, which could not be resolved by preparative HPLC. Results from binding assays show a decreased activity of the ferrocenylalanine substituted peptides compared to native SP in the following oder: [DL-Fer^8]-SP (2×10^{-2} with respect to SP) > [DL-Fer^7]-SP (3×10^{-3} relative to SP) > [DL-Fer^7, Ile^8]-SP (6×10^{-4} compared to SP).[88]

The same group also prepared ferrocenylalanine Bradykinin analogues (Scheme 5).[88] The nonapeptide

Scheme 5. Amino Acid Sequence of Bradykinin (BK, 15) and Ferrocenylalanine Analogues 16 and 17

H-Arg-Pro-Pro-Gly-X-Ser-Pro-Y-Arg-OH

X = Phe,	Y = Phe	: BK	(**15**)
X = Fer,	Y = Phe	: [Fer^5]-BK	(**16**)
X = Phe,	Y = Fer	: [Fer^8]-BK	(**17**)

Bradykinin (BK, **15**) is a tissue hormone involved in the blood clotting process. When the hormone is released from the precursor kininogen, it effects a lowering of the blood pressure via dilation of the blood vessels.[89,90] Either of the position 5 or 8 Phe residues has been substituted by DL-Fer, resulting in conjugates **16** and **17** shown in Scheme 5. Although separation of the diastereomers was accomplished by HPLC, it was not possible to assign their absolute configuration. Binding studies revealed a significant decrease of activity upon substitution of Phe by Fer. The two diastereomers of [Fer^5]-BK (**16**) and [Fer^8]-BK (**17**) showed activities relative to native BK of 7×10^{-2} and 3.5×10^{-2}, and 1.8×10^{-2} and 1.7×10^{-2}, respectively. These results show the same trend as those obtained by Couture and co-workers, who found that introduction of the bulky amino acid carboranylalanine in position 8 had a larger impact on the activity than carboranylalanine substitution of the position 5 Phe residue.[91,92] Considering the low activities of the diastereomeric [Fer]-BK analogues, it appears that the ferrocenyl moiety poorly interacts with the receptor.

The family of angiotensin II (AT II) related compounds has attracted the attention of Tartar and coworkers as well. Angiotensin II, an octapeptide having a phenylalanine in position 8, strongly increases blood pressure in mammals (**18**, Scheme 6).[93] Several

Scheme 6. Primary Structure of Angiotensin II (AT II, 18) and Derivatives 19 and 20 Thereof (Sar = *N*-Methylglycine)

H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH

AT II (**18**)

H-Sar-Arg-Val-Tyr-Ile-His-Pro-X-OH

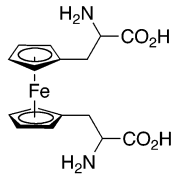
X = Phe	:	[Sar^1]-AT II (**19**)
X = Fer	:	[Sar^1, Fer^8]-AT II (**20**)

angiotensin II derivatives have found medicinal applications, such as the agonist [Asn^1, Val^5]-AT II (Hypertensin), which has been used to normalize blood pressure as quickly as possible after a shock or collapse. The antagonist [Sar^1, Val^8]-AT II (Saralasin), on the other hand, has been used for the diagnosis of AT II-dependent forms of hypertonia. Ferrocenylalanine-modified [Sar^1]-angiotensin II {[Sar^1, Fer^8]-AT II, (**20**)} has been prepared, as shown in Scheme 6.[94,95] The synthesis of **20** was performed with DL-Fer, and the diastereomers were separated by HPLC for physiological testing only. Either diastereomer showed approximately 1% activity relative to [Sar^1]-AT II (**19**) in an assay with rabbit aorta strips.[94] The diastereomeric mixture of **20** showed an activity of 6.1% relative to [Sar^1]-AT II (**19**) in a binding assay to purified bovine adrenocortical membranes.[95]

Chymotrypsin hydrolysis investigations were performed on the peptides H-Lys-Gly-Phe-Gln-Gly-OH (**21**) and H-Lys-Gly-Fer-Gln-Gly-OH (**22**).[88] Chymotrypsin belongs, together with trypsin and elastase, to the class of serine proteases and is present in the human pancreas. The high selectivity of the enzyme chymotrypsin for the scission of peptidic bonds next to the aromatic amino acids Phe, Trp, and Tyr can be rationalized by the X-ray crystal structure of bovine chymotrypsin.[96,97] Upon the action of chymotrypsin, the Phe-containing pentapeptide **21** was hydrolyzed (K_m = 3.85 mM), whereas the Fer-congener **22** was not hydrolyzed at all. Interestingly, the analogue **23** containing the unnatural amino acid cymantrenylalanine (cymantrene = $Mn(Cp)(CO)_3$) was hydrolyzed, albeit with lower affinity than the Phe derivative (K_m = 37 mM, but unchanged V_{max}).[88]

The amino acid ferrocenylalanine (Fer, **2**) alone also has been subjected to biological tests. In a bacterial growth assay with the phenylalanine requiring bacterium *Leuconostoc mesenteroides*, DL-Fer was tested for its ability to (1) support bacterial growth in the absence of Phe and (2) inhibit bacterial

Scheme 7. Molecular Structure of 1,1′-Ferrocenylbisalanine (24)



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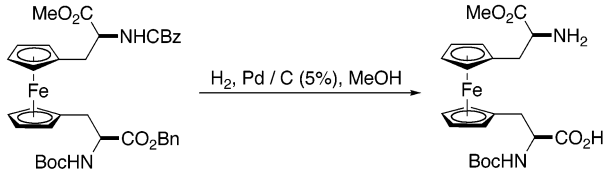
Scheme 8. Synthesis of an Optically Pure 1,1′-Ferrocenylbisalanine Derivative 27 Containing a Free Amino and a Free Carboxylic Acid Moiety



26 27

growth in the presence of Phe. DL-Fer did not support growth of *L. mesenteroides* in the absence of Phe, nor was it able to inhibit the growth of this bacterium in the presence of Phe.[98,99] Furthermore, DL-Fer was tested for toxicity against Chinese hamster ovarian (CHO) cells.[99] Only at relatively high concentrations of 0.45 mM or higher, was DL-Fer toxic against CHO cells. DL-Fer was also tested for its function to serve as a substrate or inhibitor of phenylalanine hydroxylase and aromatic L-amino acid decarboxylase. For phenylalanine hydroxylase, DL-Fer was a noncompetitive inhibitor (K_i = 0.89 mM) with respect to L-Phe and a mixed inhibitor with respect to the cofactor ($DMPH_4$).[98,99] DL-Fer was found to be a competitive inhibitor (K_i = 7.2 mM) of aromatic L-amino acid decarboxylase with respect to phenylalanine.

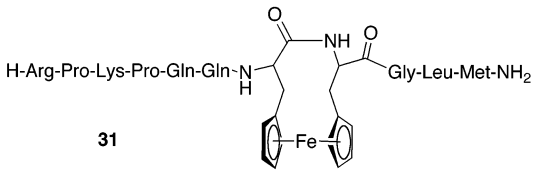
2.1.2. 1,1′-Ferrocenylbisalanine and Its Conjugates

In addition to ferrocenylalanine, also the difunctionalized derivative 1,1′-ferrocenylbisalanine (**24**, Scheme 7) has been reported.[74,100−105] The methods for obtaining **24** in optically pure form are identical to those for ferrocenylalanine: (1) Pd(0)-catalyzed cross-coupling of 1,1′-diiodoferrocene (**25**) with the serine-derived organozinc reagent **8** shown in Scheme 2[74] and (2) asymmetric hydrogenation of suitable bis-didehydro amino acid derivatives.[101−104] With the latter method, an enantiomeric excess higher than 99% could be achieved.[103,104] Via a sophisticated synthetic route, Frejd and co-workers synthesized an enantiomerically pure 1,1′-ferrocenylbisalanine derivative **26** that contains orthogonal protecting groups (Scheme 8).[102−104,106]

Optically pure **26** was conveniently transformed by hydrogenation into *S,S*-**27**, having a free amino group and a free carboxylic acid moiety (Scheme 8). When **27** was reacted with the peptide coupling reagent PyAOP, a mixture of the 1,1′-ferrocenophane lactam **28** and two macrocyclic peptides (dimer **29** and trimer **30**) was obtained (Scheme 9).[106] The exact ratio between these compounds depends strongly on the reaction conditions.

Frejd and co-workers used lactam **28** as a structural H-Phe-Phe-OH mimetic and incorporated it into a peptide.[103] This resulted in a conjugate **31**, which was expected to function as a structural mimic of substance P (Scheme 10). However, CD spectroscopic

Scheme 10. Structural Mimic of Substance P, with the 1,1′-Ferrocenophane Serving as a Substitute for the Dipeptide Phe-Phe



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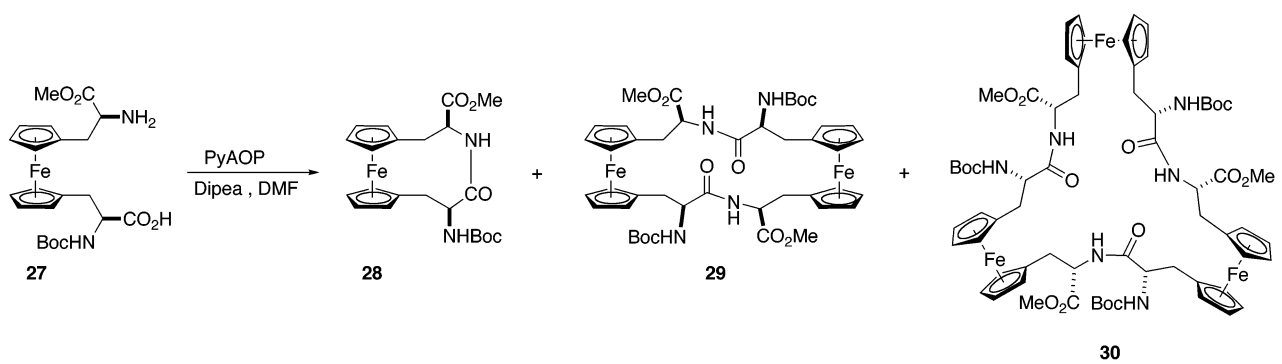
studies indicated that the constraints imposed by the 1,1′-ferrocenophane prohibited the peptide to adopt the characteristic α-helical secondary structure found for native SP in a biomimetic SDS (sodium *n*-dodecyl sulfate) micellar environment. According to the authors, the results from biological activity tests of this SP mimic will be published in due course.[103] The outcome of these tests will be interesting, especially in comparison to the activities determined for the Fer-SP analogues described above.[88]

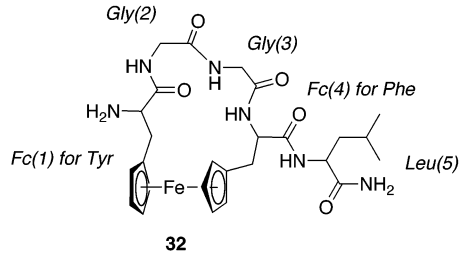
In another interesting paper, Frejd and co-workers prepared a [Leu^5]-enkephalin mimetic in which the H-Tyr-Gly-Gly-Phe subunit has been replaced by a Gly-Gly looped 1,1′-ferrocenylbisalanine residue (**32**, Scheme 11).[104] The ferrocene rings of this constrained compound constitute a substitute for the aromatic phenol (Tyr) and phenyl (Phe) rings. NMR spectroscopic studies revealed this conjugate to possess a hydrogen bond between the Fer CO group and the

Scheme 9. Lactamization of 27 to Yield Macrocycles 28−30



27 28 29 30

Scheme 11. Structural Mimic of [Leu^5]-Enkephalin (9) with the 1,1′-Ferrocenophane



NH moiety of the other Fer ring. This conformation represents a model for native [Leu^5]-enkephalin in the single-bend conformation, which is stabilized by a β-turn.[107,108] Unfortunately, binding studies of this compound to the opioid receptor have as yet not been reported.

2.1.3. Other Ferrocenyl-Based Amino Acids

At this stage, it should be noted that several other ferrocenylalanine analogues have been reported. These include 1,2-ferrocenylbisalanine **33**,[100] which was readily obtained as the bis(*N*-formyl) derivative but decomposed upon deprotection (Scheme 12). The β-amino acids ferrocenyl-β-alanine (**34**) and 1,1′-ferrocenylbis-β-alanine (**35**) could be synthesized in enantiomerically pure form (Scheme 12).[109] No attempts were made to hydrolyze the methyl esters and obtain the free amino acids. Furthermore, some derivatives have been synthesized which formally have an amino acid type structure: 1,1′-ferrocenylbisglycine,[110] ferrocenylenebisvaline,[111] and a 1,1′-functionalized ferrocene with *N*,*N*-dibenzylalanine-like moieties.[112]

The synthesis of the simplest ferrocene-containing amino acid 1′-aminoferrocene-1-carboxylic acid (**36a**, Fca, R = R′ = H, Scheme 12) has been reported. A first preparation by lithiation of 1′-amino-1-bromoferrocene, followed by quenching with solid carbon dioxide, did not yield pure **36a**.[113] At about the same time, a synthesis which yielded **36a** in rather low yield was published,[114] along with a careful structural comparison of derivatives of **36a** in the solid state and in solution. The synthesis was recently improved by Heinze and Schlenker.[115] Rapić and co-workers were able to obtain this very interesting compound starting from ferrocene-1,1′-dicarboxylic acid along with several protected derivatives.[116] Several protected derivatives of **36** were also prepared (Scheme 12). The X-ray single-crystal structures of the *N*-acetyl methyl ester **36b** (R = Me, R′ = Ac, Ac-Fca-OMe)[117] and the *N*-Boc-protected **36c** (R = H, R′ = Boc, Boc-Fca-OH) were published.[118] Starting from **36b**, a number of amino acid and peptide derivatives were prepared in which **36** serves as one amino acid. These peptides show interesting intramolecular hydrogen bonds. In collaboration with Rapić's group, we have published the first oligopeptide derivative of **36**.[119] Figure 1 shows an ORTEP plot of the compound Boc-Ala-Fca-Ala-Ala-OMe (**37**), which is formally a tetrapeptide. Peptide **37** was prepared in solution by modified peptide coupling reactions and purified by thin-layer chromatography. The configuration of the peptide strands is stabilized by two intramolecular hydrogen bonds, which enforce a P-helical conformation at the metallocene. As shown by CD spectroscopy, this conformation is preserved in solution and very stable indeed. In contrast to the compounds discussed in section 2.2.2 below, peptides such as **37** extending from the C- and N-termini of **36** form *antiparallel* sheets. As such, **36a** is not just an organometallic amino acid but also the first "real" organometallic turn mimetic.

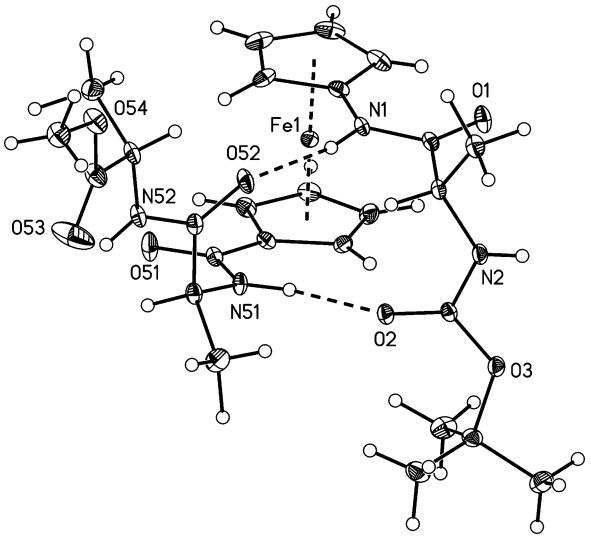
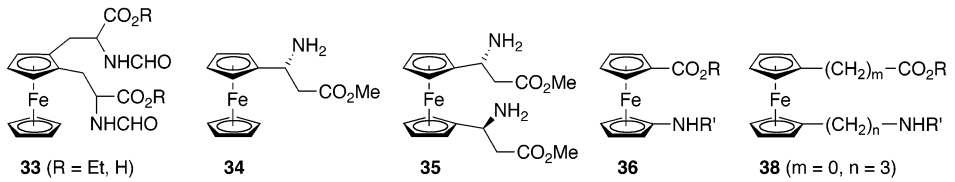


Figure 1. Solid-state structure of the tetrapeptide Boc-Ala-Fca-Ala-Ala-OMe **37** containing the unnatural 1′-aminoferrocene-1-carboxylic acid (Fca).[119]

Other organometallic amino acids such as **38a** and **39a** (R = R′ = H in both cases) were reported by the same group.[120] Both compounds were prepared in several steps and could be comprehensively characterized. The X-ray single-crystal structures of **38b** (R = Me, R′ = Ac) and **39d** (R = Me, R′ = Boc) were reported, along with the preparation of several other derivatives with varying protecting groups. These compounds should prove to be flexible building blocks for peptides with a ferrocenyl unit incorporated into the peptide chain.

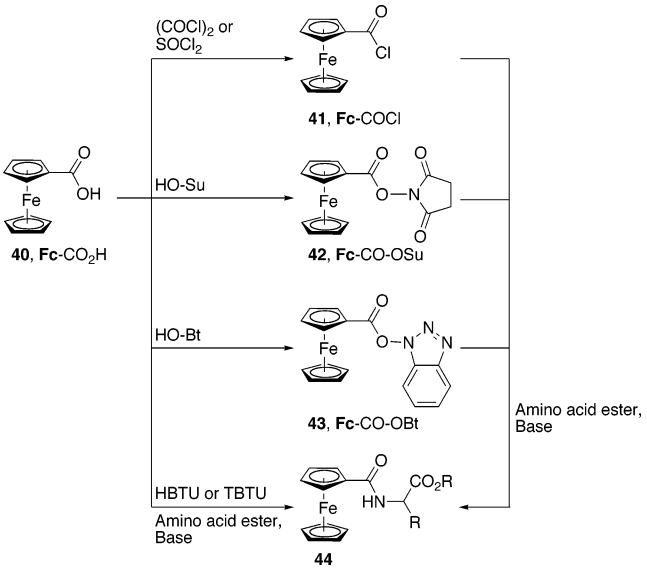
Scheme 12. Ferrocene-Based Amino Acids



2.2. Amide Formation with Ferrocenecarboxylic Acid Derivatives

The most extensively explored way to couple the ferrocene moiety to amino acids and peptides is via amide formation between ferrocenecarboxylic acid (**40**) and the terminal amino group. The first amino acid and dipeptide derivatives of this kind, Fc-CO-Gly-OMe, Fc-CO-Gly-OH, and Fc-CO-Gly-Leu-OEt, were reported by Schlögl as early as 1957.[43] To make coupling of a carboxylic acid with an amino group possible under mild conditions at room temperature, the acid needs to be activated. There are three strategies to activate this carboxylic acid (Scheme 13): (1) transformation into the acid chloride **41** with

Scheme 13. Synthetic Possibilities to Obtain Ferrocene Carboxylic Amide Derivatives of Amino Acids and Peptides



oxaloyl chloride or thionyl chloride,[43,121] (2) transformation of the acid **40** into the succinimid **42** or benzotriazole ester **43**, with the use of DCC or EDC in conjunction with *N*-hydroxysuccinimid (HOSu) or 1-hydroxybenzotriazole (HOBt),[122] or (3) activation of the acid in situ by HBTU or TBTU.[123] From a practical point of view, the third method is preferred because of the following reasons: (1) it is a one-pot procedure, with the activation occurring in situ after mixing the carboxylic acid and amine components with TBTU or HBTU in the presence of a base like NEt_3; (2) the coupling requires short reaction times of <45 min; (3) it is not necessary to use dry solvents; commercial grade solvents, preferably DMF or MeCN, can be employed; and (4) the side-products originating from the coupling reagents can be readily removed by an appropriate extractive workup, yielding products of good purity without further purification.[123,124] Beck and co-workers reported another interesting method to transform ferrocenoylamino acids into the corresponding dipeptide derivatives via oxazolone formation with the use of a carbodiimide.[125]

2.2.1. Amides from Ferrocenecarboxylic Acid

Up to now, a large amount of monosubstituted ferrocenoylamino acid and peptide conjugates of defined molecular structure are known; an overview is given in Table 1. Several of these derivatives have been structurally characterized by X-ray crystallography (see Table 1). In the solid state, hydrogen bond interactions are a dominant feature, and zigzag,[126,127] sheetlike,[128] and helical packing arrangements[128,123] have been observed. In solution, most of these derivatives do not appear to have an ordered structure. Exceptions, however, are Fc-CO-Pro-Pro-Phe-OH and Fc-CO-$(Pro)_x$-OBn with $x = 2-4$, compounds reported by Kraatz and co-workers.[129] The former, an ORTEP plot of which is depicted in Figure 2, reveals a structural motif characteristic for a

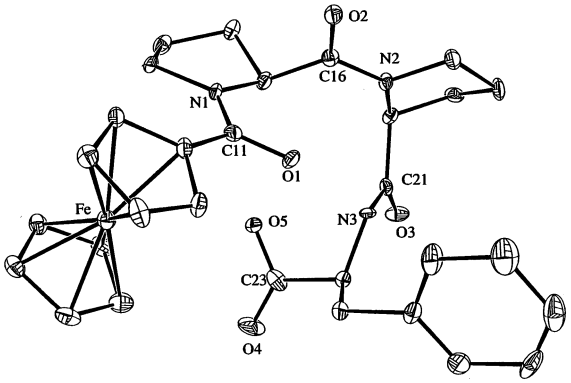


Figure 2. ORTEP plot of the Fc-CO−Pro-Pro-Phe-OH. Reprinted (with modifications) with permission from ref 129. Copyright 1999 Elsevier.

β-turn in proteins. The two Pro residues are connected in a *cis*-fashion, and a strong hydrogen bond is present between the proton on the Phe amide nitrogen atom N3 and the ferrocenyl O atom (N···O contact = 2.853 Å). NMR spectroscopic investigations confirm that the observed solid-state conformation is maintained in MeCN-d_3 solution.[129]

The X-ray crystal structure of Fc-CO-$(Pro)_4$-OBn is shown in Figure 3.[129] The amide bonds between

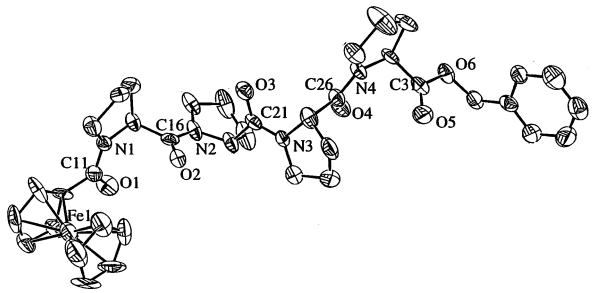


Figure 3. ORTEP plot of Fc-CO(-Pro)$_4$-OBn. Reprinted (with modifications) with permission from ref 129. Copyright 1999 Elsevier.

the Pro residues are *trans*-configured, and the compound has a left-handed helical conformation characteristic of polyproline II.[130,131] X-ray crystal structures of the related compounds Fc-CO-$(Pro)_3$-OBn and Fc-CO-$(Pro)_2$-OBn also show a left-handed helical orientation, with *trans*-configured proline linkages.[129] NMR spectroscopic investigations revealed that the solid-state conformation of these proline compounds is maintained in $CDCl_3$ and MeCN-d_3; no cross-peaks indicative of *cis*-proline linkages were observed in the NOESY NMR spectra.

Table 1. Overview of the Reported Ferrocenoylamino Acid and Peptide Derivatives

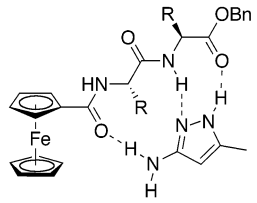
compound	ref	compound	ref
	Ferrocenoylamino Acid Derivatives		
Fc-CO-Gly-OMe	144[a]	Fc-CO-Ala-OMe	125
Fc-CO-Gly-OEt	43, 122, 125	Fc-CO-Ala-OEt	125
Fc-CO-Gly-OBn	145[a]	Fc-CO-Ala-OBn	122[a]
Fc-CO-Gly-OH	43, 125	Fc-CO-Ala-OH	125
Fc-CO-Gly-OSu	146[b,c]	Fc-CO-Asp-OH	136[a]
Fc-CO-Cys(SBn)-OMe	122[c]	Fc-CO-Asp(OBn)-OBn	136
Fc-CO-Met-OEt	147	Fc-CO-Pro-OH	148, 129[a]
Fc-CO-Pro-OBn	122	Fc-CO-Pro-OMe	121
Fc-CO-Phe-OMe	125, 121, 123, 149[c]	Fc-CO-Phe-OH	125, 148
Fc-CO-Phe-OtBu	146	Fc-CO-Phe-OBn	122
Fc-CO-Tyr-OBn	122	Fc-CO-Val-OMe	121, 150[a]
Fc-CO-DL-Leu-OH[d]	148	Fc-CO-DL-Val-OH	148
Fc-CO-Lys(N_ϵ-CO-Fc)OH[d]	148	Fc-CO-Glu(OBn)-OBn	122[a]
Fc-CO-His-OMe	151	Fc-CO-His(N_ϵ-CO-Fc)-OMe	151[a]
[Fc-CO-Gly-NHC_2H_4S]$_2$	133	[Fc-CO-Pro-NHC_2H_4S]$_2$	132
[Fc-CO-Ala-NHC_2H_4S]$_2$	152[c]		
	Ferrocenoyl Dipeptide Derivatives		
Fc-CO-Gly-Gly-OEt	125, 134, 128[a]	Fc-CO-Gly-Gly-OMe	149[c]
Fc-CO-Gly-Ala-OMe	125	Fc-CO-Gly-Pro-OEt	153
Fc-CO-Ala-Ala-OBn	134, 128	Fc-CO-Ala-Ala-OMe	125
Fc-CO-Ala-Pro-OEt	126,[a] 127, 154	Fc-CO-Ala-Phe-OEt	149[c]
Fc-CO-Ala-Phe-OMe	123[a]	Fc-CO-Asp(OBn)-Asp(OBn)-OBn	136
Fc-CO-Asp(OBn)-Glu(OEt)-OEt	136	Fc-CO-Asp(OBn)-Cys(SBn)-OMe	136
Fc-CO-Leu-Phe-OMe	134, 128[a]	Fc-CO-Pro-Gly-OEt	153
Fc-CO-Pro-Pro-OBn	129[a]	Fc-CO-Gly-Leu-OEt	43
Fc-CO-Ala-Pro-NHPy[a,e,f]	155	Fc-CO-Phe-Phe-OMe	128, 149[c]
Fc-CO-Phe-Leu-OBn	149[c]	Fc-CO-Phe-Ser-OEt	149[c]
Fc-CO-Val-Phe-OMe	128	Fc-CO-Val-Val-OMe	128
Fc-CO-Leu-Leu-OEt	149[c]	Fc-CO-Leu-Leu-OMe	128
[Fc-CO-Pro-Pro-NHC_2H_4S]$_2$	132		
	Ferrocenoyl Tri-, Tetra-, and Oligopeptide Derivatives		
Fc-CO-Pro-Pro-Pro-OBn	129[a]	Fc-CO-Pro-Pro-Phe-OH	129[a]
Fc-CO-Gly-Phe-Leu-OH[c]	148	Fc-CO-Pro-Pro-Pro-OBn	129[a]
Fc-CO-Gly-Gly-Tyr(OBn)-Arg(NO_2)-OMe	156	Fc-CO-Gly-Gly-Tyr(OBn)-Arg(NO_2)-OH	156
Fc-CO-Gly-Gly-Tyr-Arg-OH	156	Fc-CO-(substance P)	140[g]
Fc-CO-Pro-Gln-Phe-Phe-Gly-Leu-Met-NH_2[c]	148[h]	[Fc-CO-$(Pro)_x$-NHC_2H_4S]$_2$	132[i]
Fc-CO-$(Gly)_4$-NHC_2H_4SH	157	[Fc-CO-$(Pro\text{-}Pro\text{-}Gly)_y$-$NHC_2H_4S$]$_2$	152[c,j]

[a] X-ray crystal structure presented in this reference. [b] OSu = succinimidyl. [c] Not well characterized. [d] Racemic mixture used. [e] NHPy = amide of 2-aminopyridine. [f] Also the complexation of $PdCl_2$ to the pyridine nitrogen atoms has been reported in this paper. [g] Substance P is an undecapeptide (see Schemes 4 and 15). [h] This is the 4−11 amino acid fragment of substance P. [i] x = 3−6. [j] y = 1−3.

Galka and Kraatz also reported a series of disulfide-bridged prolyl cystamine ferrocenoyl compounds of the general formula Fe(C_5H_4-CO-$(Pro)_n$-$NHC_2H_4S)_2$, with $n = 0-6$.[132] Self-assembled monolayers on a gold electrode were prepared from these compounds, and the electron-transfer properties between the ferrocenoyl moiety and the surface of the gold microelectrode were investigated. A through-bond mechanism of the electron-transfer process is suggested on the basis of a significant deviation from Marcus-type behavior, although k_{ET} shows a distance dependence.

Kraatz et al. also prepared the glycyl cystamine derivative Fe(C_5H_4-CO-Gly-$NHC_2H_4S)_2$.[133] This compound crystallizes in a supramolecular helical assembly consisting of two different helices. In two papers, the same group studied the interaction of several ferrocenoyldipeptides (Fc-CO-Gly_2-OEt, Fc-CO-Ala_2-OBn, Fc-CO-Phe_2-OMe, Fc-CO-Leu_2-OMe, Fc-CO-Val_2-OMe, Fc-CO-Leu-Phe-OMe, and Fc-CO-Val-Phe-OMe) with 3-aminopyrazole (Apzl), 3-amino-5-methylpyrazole (3-AMP), and 3-trifluoroacetylamido-5-methylpyrazole (3-TFAc-AMP).[134,128] In solution, these compounds were found to form 1:1 associates stabilized by three hydrogen bonds, as shown schematically for **45** in Scheme 14. From the results of

Scheme 14. Hydrogen Bond Interaction between Fc-CO-Ala_2-OBn and 3-Amino-5-methylpyrazole (3-AMP) in Solution



45

^{1}H NMR titration experiments, the binding constants between these pyrazole derivatives and the dipeptides in $CDCl_3$ were determined to range from 8 to 27 M^{-1}. These values correspond to moderate binding energies of 5−7 kJ mol^{-1}. In solvents of higher polarity such as MeCN-d_3 and acetone-d_6, the binding constants were found to be <5 M^{-1}.

Recently, Kraatz presented an overview of his extensive work on ferrocenoylpeptides.[135] The electrochemical properties of ferrocenoylpeptides were also discussed in that account. Redox potential

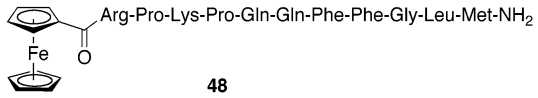
determinations on ferrocenoylamino acids and ferrocenoylpeptides performed by different groups were compared. However, the outcome and conclusions should be taken with the appropriate care because each group references their redox potential in a different way. Kraatz defines FcH/FcH^+ as +450 mV versus SCE,[122] whereas the Hirao group measures the redox potential versus Ag/Ag^+ and determined the FcH/FcH^+ couple in an external measurement to be +0.51 V.[127] This underlines the need for a consistent method to solve this general problem, and we suggest to use the FcH/FcH^+ couple as an internal standard, that is, adding a small amount of ferrocene at the end of each electrochemical investigation to determine the exact redox potential under the conditions used.[123] This gives very reliable values relative to FcH/FcH^+ which are easily reproducible and independent of solvent, experimental setup, and conditions. This procedure is also recommended by IUPAC.

An interesting result that is certainly reliable is the observed redox potential shift of the Fc/Fc^+ transition toward less positive potentials when the attached peptide becomes more helical. On going from Fc-CO-Pro-OH to Fc-CO-Pro_4-OBn, a decrease of the redox potential of about 30 mV occurs.[129,135] In another interesting paper, Kraatz and co-workers measured the redox potential of ferrocenoyl dipeptides in a large variety of solvents, concluding that the redox potential could be correlated with the hydrogen donor ability α of the Kamlet−Taft formalism.[136]

The peptide derivatives Fc-CO-Phe-OMe (**46**) and Fc-CO-Ala-Phe-OMe (**47**) were subjected to ^{57}Fe Mössbauer spectroscopic investigations.[123] The values for the isomer shift and quadrupole splitting are in the range reported for several other ferrocene derivatives that have electron-withdrawing substituents on the Cp ring[137,138] and resemble those of ferrocene−peptide nucleic acid (PNA) derivatives.[139] The peptide fragment appears to have little influence on the electric field gradient of the iron nucleus.

The peptide derivatives presented thus far in this section have all been synthesized via peptide synthesis methods in solution. However, a ferrocenoyl-labeled derivative **48** of the undecapeptide substance P has been prepared via classical Merrifield solid-phase synthesis methods (Scheme 15).[140] In contrast

Scheme 15. Structure of the Ferrocenyl Substance P Conjugate 48 Obtained via Merrifield Solid-Phase Synthesis



48

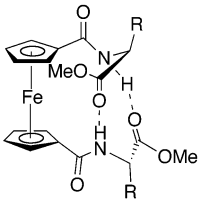
to the ferrocenylalanine derivatives presented in section 2.1 (Scheme 4), no serious oxidation or decomposition was observed upon cleavage of the ferrocenoyl conjugate **48** from the resin with HF. The higher robustness of **48** has been attributed to the increase in oxidation potential of the ferrocene moiety, on account of the electron density withdrawing effect of the amide. Interestingly, the spasmogenic potency of **48** was identical within the experimental error to that of native SP (0.93 ± 0.10 relative to native SP), determined by a guinea pig ileum assay.

At this stage, it should be noted that a 21-mer helical peptide containing six ferrocenoyl groups attached to lysine-N_ϵ-amino groups has been prepared.[141,142] The idea behind the synthesis of the conjugate was to spectroscopically investigate whether the conformation of the parent peptide is preserved upon derivatization. The spectroscopic results showed the conjugate to adapt a helical conformation similar to that of the unmodified peptide. Another paper reported on the reaction of a L-lysine polymer of MW 126.2 kDa with **41**.[143] In this case, the conjugate was obtained with 6% of the lysine residues carrying a ferrocenoyl tag. In the same paper, a 52.1 kDa lysine copolymer, having the amino acid molar ratio Lys/Ala/Glu/Tyr of 34:45:14:7, was reacted with **41**. This resulted in derivatization of 14% of the lysine NH_2 groups. These two water-soluble ferrocenoyl polymers were used for the construction of an amperometric glucose-sensing electrode, by immobilizing them together with the enzyme glucose oxidase (GOD) on the surface of a glassy carbon electrode.

2.2.2. Amides from Ferrocene-1,1′-dicarboxylic Acid

In addition to monosubstituted amino acid derivatives, a large number of 1,1′-disubstituted amino acid derivatives have been prepared. An overview of these is given in Table 2. In 1996, Herrick et al. found that compounds of general formula $Fe(C_5H_4\text{-CO-Aaa-OMe})_2$, with Aaa other than Pro, have an ordered structure in CH_2Cl_2 and $CHCl_3$ (**49**, Scheme 16).[121]

Scheme 16. Ordered Conformation Observed for $Fe(C_5H_4\text{-CO-Aaa-OMe})_2$ in CH_2Cl_2 and $CHCl_3$ Solution



49

This ordered structure is comprised of two symmetrically equivalent hydrogen bonds between the amide NH and the methyl ester carbonyl oxygen atom of another strand. From the rationale of the poor hydrogen bond acceptor properties of methyl ester carbonyl groups, this conformation appeared at first somewhat doubtful to us. However, we were able to confirm that the phenylalanine derivative $Fe(C_5H_4\text{-CO-Phe-OMe})_2$ (**50**) indeed adapts such a conformation in CH_2Cl_2 and $CHCl_3$.[123] In contrast, the analogous (charged) cobaltocenium compound was found to exist as a mixture of this hydrogen-bonded conformation and a non-hydrogen-bonded form at room temperature in CH_2Cl_2 and $CHCl_3$. This was concluded from the presence of two C=O stretching vibrations in the solution IR spectrum in CH_2Cl_2 at 3365 and 3404 cm^{-1} (cf. the ferrocene analogue displays only one stretching vibration in CH_2Cl_2 at 3380 cm^{-1}).

Table 2. Overview of the Reported 1,1′-Bisamino Acid and 1,1′-Bispeptide Derivatives

compound	ref	compound	ref
	1,1′-Bisamino Acid Ferrocene Derivatives		
$Fe(C_5H_4$-CO-Gly-OEt$)_2$	173[a,f]	$Fe(C_5H_4$-CO-Gly-OH$)_2$	173[a,f]
$Fe(C_5H_4$-CO-Gly-$NH_2)_2$	158[b]	$Fe(C_5H_4$-CO-Pro-OMe$)_2$	121, 168[b]
$Fe(C_5H_4$-CO-Pro-OBn$)_2$	168	$Fe(C_5H_4$-CO-Phe-OMe$)_2$	121, 123[b]
$Fe(C_5H_4$-CO-Val-OMe$)_2$	148, 150[b]	$Fe(C_5H_4$-CO-Cys(SEt)-OMe$)_2$	164, 165
$Fe(C_5H_4$-CO-Gly-$NHC_2H_4S)_2$	166[b]	$Fe(C_5H_4$-CO-Ala-$NHC_2H_4S)_2$	166
$Fe(C_5H_4$-CO-Val-$NHC_2H_4S)_2$	166	$Fe(C_5H_4$-CO-Leu-$NHC_2H_4S)_2$	166
	1,1′-Bispeptide Ferrocene Derivatives		
$Fe(C_5H_4$-CO-Gly-Gly-OMe$)_2$	125	$Fe(C_5H_4$-CO-Gly-Ala-OMe$)_2$	125
$Fe(C_5H_4$-CO-Gly-Leu-OEt$)_2$	126,[b] 154	$Fe(C_5H_4$-CO-Gly-Phe-OMe$)_2$	125
$Fe(C_5H_4$-CO-Gly-Pro-OEt$)_2$	153	$Fe(C_5H_4$-CO-Gly-Phe-OEt$)_2$	154
$Fe(C_5H_4$-CO-Ala-Pro-OEt$)_2$	126,[b] 127, 154	$Fe(C_5H_4$-CO-D-Ala-D-Pro-OEt$)_2$	154
$Fe(C_5H_4$-CO-Ala-Pro-OMe$)_2$	127[b]	$Fe(C_5H_4$-CO-Ala-Pro-OPr$)_2$	127[b]
$Fe(C_5H_4$-CO-Ala-Pro-OBn$)_2$	127[b]	$Fe(C_5H_4$-CO-Pro-Gly-OEt$)_2$	153
$Fe(C_5H_4$-CO-Ala-Phe-OMe$)_2$	123[b]	$Fe(C_5H_4$-CO-Pro-Pro-OBn$)_2$	168[c]
$Fe(C_5H_4$-CO-Ala-Pro-NHPy$)_2$	160[b,d]	$Fe(C_5H_4$-CO-Ala-Pro-NHPy$)_2PdCl_2$	160[b,d]
$Fe(C_5H_4$-CO-Ala-Pro-NHPy$)_2$·CA	161[b,e]	$Fe(C_5H_4$-CO-Pro_3-OBn$)_2$	168[c]
$Fe(C_5H_4$-CO-Pro_4-OBn$)_2$	168[c]	Boc-$NHCH_2CH_2NHCO$-$(C_5H_4)Fe(C_5H_4)$-CO-Ala-$(Leu\text{-}Aib)_8$-lipoamide	174[e]

[a] Not well characterized. [b] X-ray crystal structure reported in this reference. [c] Also the derivative $Fe(C_5H_4$-CO-Pro_3-OBn$)(C_5H_4$-CO-OBt) is isolated from the same reaction. [d] NHPy = amide of 2-aminopyridine. [e] CA = (1*R*,3*S*)-camphoric acid; Aib = aminoisobutyric acid, also known as dimethylglycine; lipoamide = amide of lipoamine. [f] Those two compounds were recently comprehensively characterized by Kraatz and co-workers, including X-ray single-crystal structures.[175]

Interestingly, the ordered conformation shown for **49** in Scheme 16 is observed for the valine derivative $Fe(C_5H_4$-CO-Val-OMe$)_2$ (**51**) in the crystalline state, although the hydrogen bond interactions are quite weak (N···O contacts of 3.25 Å).[150] In contrast, the bulkier Phe-OMe derivative **50** has a different conformation in the solid state (see Figure 4), although

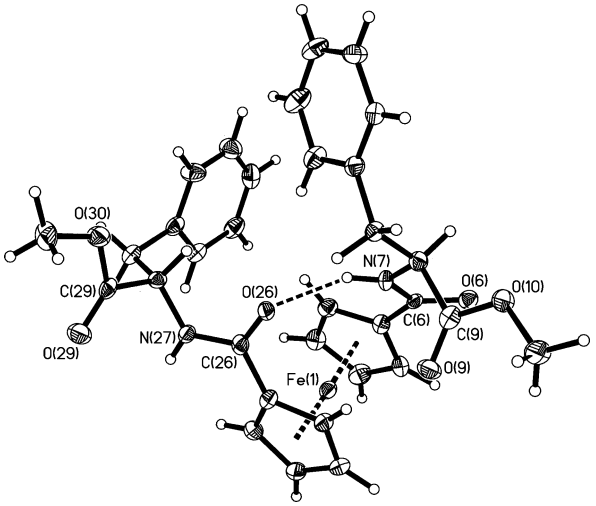


Figure 4. Solid-state structure of $Fe(C_5H_4$-CO-Phe-OMe$)_2$ (**50**). Reprinted (with modifications) with permission from ref 123. Copyright 2003 The Royal Society of Chemistry.

it adapts the conformation shown in Scheme 16 in solution. In the solid state, **50** forms one intramolecular hydrogen bond and an intermolecular one, assembling in a right-handed helix.[123] Perhaps the difference between **50** and **51** is due to T-stacking of the aromatic rings in the former.

The difference in hydrogen bond acceptor strengths between amide carbonyl moieties and ester carbonyl groups is nicely illustrated by the X-ray crystal structures of **51** and $Fe(C_5H_4$-CO-Gly-$NH_2)_2$ (**52**).[150,158] Both display the ordered conformation with the two symmetrical hydrogen bonds, but the N···O contacts are considerably shorter for the glycinamide compound **52** compared to the valine methyl ester **51** (2.88 vs 3.25 Å).

In addition to the 1,1′-disubstituted amino acid derivatives, also compounds have been prepared in which each Cp ring is substituted by a dipeptide ester; an overview of these is given in Table 2. Hirao and co-workers were the first to report and to investigate compounds of general composition $Fe(C_5H_4$-CO-Ala-Pro-OR$)_2$ (**53**), with (R = Me (**a**), Et (**b**), *n*-Pr (**c**), Bn (**d**)).[126,127] As a representative example, the X-ray crystal structure of **53a** is depicted in Figure 5.[127] The structure reveals an ordered conformation related to **49** shown in Scheme 16, having intramolecular hydrogen bonds between the Ala-NH and the Ala-CO of another strand, with N···O contacts of 2.91 and 3.04 Å.

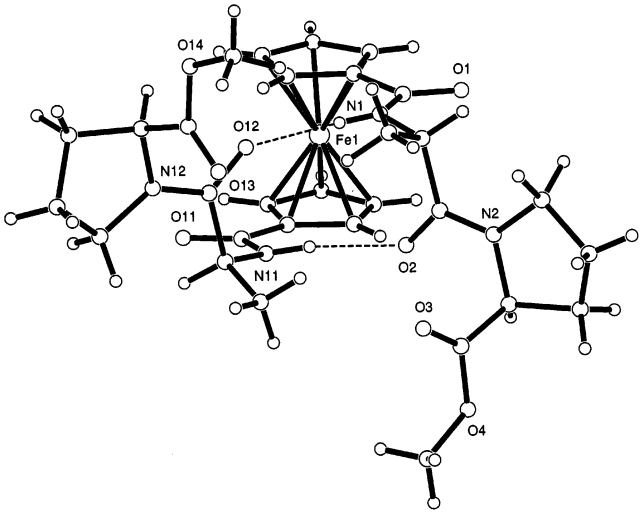


Figure 5. Solid-state structure of $Fe(C_5H_4$-CO-Ala-Pro-OMe$)_2$ (**53a**). Reprinted (with modifications) with permission from ref 127. Copyright 1999 Elsevier.

All these derivatives with varying R groups display a similar type of ordered structure in the solid state as well as in solution, even in polar media, such as MeCN and $CDCl_3$/DMSO-d_6 (9:1 v/v) mixtures. This was derived by combining the results from NMR, IR, and CD spectroscopic investigations.[127] In Figure 6,

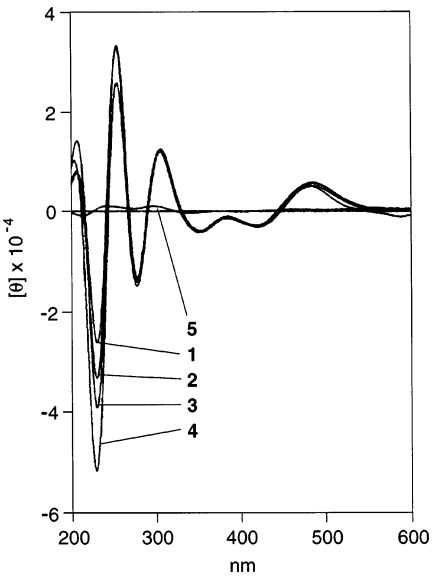


Figure 6. CD spectra of Fe(C_5H_4-CO-Ala-Pr−OR)$_2$ (**53**) (with R = Et, **1**; Me, **2**; *n*-Pr, **3**; and Bn, **4**) and Fc-CO-Ala-Pro-OEt (**5**) in MeCN (0.1 mM). Reprinted with permission from ref 127. Copyright 1999 Elsevier.

the CD spectra of Fc-CO-Ala-Pro-OEt (**54**) and **53a**−**d** in MeCN are shown.[127] The Cotton effects owing to the ferrocene chromophore are of much higher intensity for the 1,1′-disubstituted compounds **53** than for the monosubstituted ferrocenoyl derivative **54**, which confirms that the former group of compounds has an ordered structure. Hirao and co-workers prepared and structurally characterized the D-amino acid-containing compound Fe(C_5H_4-CO-D-Ala-D-Pro-OEt)$_2$.[154] The fact that this compound is in an enantiomeric relationship to Fe(C_5H_4-CO-Ala-Pro-OEt)$_2$ (**53b**) has been nicely illustrated by CD spectroscopy: Cotton effects of identical intensity, but opposite sign, were observed.

It is noteworthy that the compound Fe(C_5H_4-CO-Gly-Pro-OEt)$_2$ (**55**) has the same type of arrangement as the above-mentioned Ala-Pro derivatives **53** in the solid state as well as in solution.[153] In addition to the compounds **53** and **55**, which possess one NH group per peptide strand, peptide derivatives with two NH amide moieties have been prepared. These include Fe(C_5H_4-CO-Gly-Leu-OEt)$_2$ (**56**), Fe(C_5H_4-CO-Gly-Phe-OEt)$_2$ (**57**),[154] and Fe(C_5H_4-CO-Ala-Phe-OMe)$_2$ (**58**).[123] The amide NH moieties of the second amino acid are not orientated in such a way to allow the formation of intramolecular hydrogen bonds. Instead, these NH groups are involved in intermolecular hydrogen bond interactions in the solid state, resulting in supramolecular assemblies. X-ray crystallographic analysis revealed **56** to assemble in a chainlike arrangement throughout the lattice.[154] Both **57** and **58**, on the other hand, were found to form 14-membered hydrogen-bonded rings, as depicted in Figure 7 for the latter.[123,154]

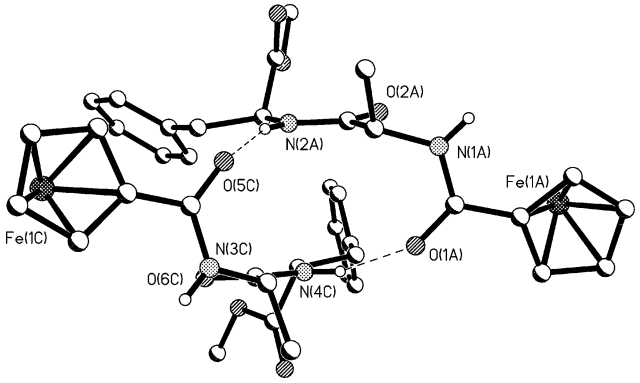
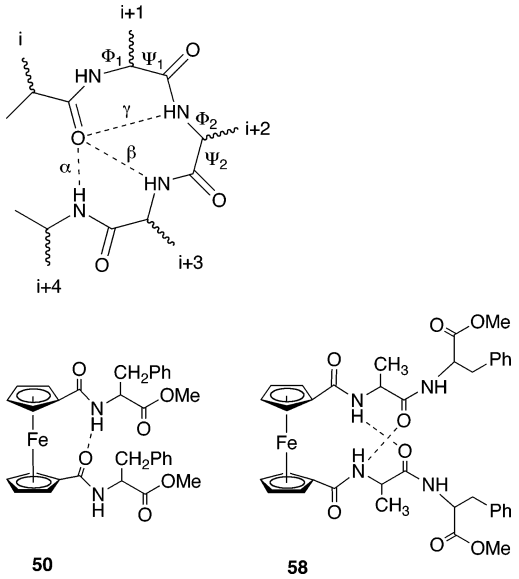


Figure 7. Solid-state structure of Fe(C_5H_4-CO-Ala-Phe-OMe)$_2$ (**58**), depicting the 14-membered hydrogen-bonded ring. Reprinted (with modifications) with permission from ref 123. Copyright 2003 The Royal Society of Chemistry.

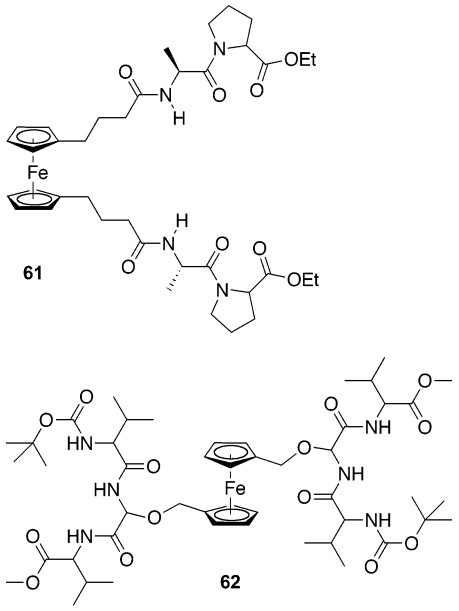
To some extent, the structures discussed above resemble turn structures in peptides. A typical peptide turn structure is depicted in Scheme 17. Ring

Scheme 17. Intramolecular Hydrogen Bonds in 50 and 58 in Comparison to Naturally Occurring Peptide Turn Structures (Top)



constraints by hydrogen bonds define γ-turns (7-membered ring) or β-turns (10-membered ring), whereas 13-membered rings are present in α-helices. Before and after the turn, the peptide extends as antiparallel strands, often in β-sheets. In all peptide derivatives of ferrocene-1,1′-dicarboxylic acid a parallel orientation of the peptide strands is enforced. Although these compounds may not be regarded as true β-turn mimetics,[121] topological similarities remain. On the basis of this consideration, we have suggested a nomenclature for such parallel turn mimetics,[123] and this nomenclature is exemplified by the solid-state structures of **50** and **58** (Scheme 17). For **50**, an 8-membered ring is formed across the hydrogen bond (H−N−$C_{C=O}$−$C_{Cp-ipso}$−Fe−$C_{Cp-ipso}$−$C_{C=O}$−O). The hydrogen bond is formed between the *i* (C=O) and *i* + 2 (NH) amino acids. This type of turn we denote pseudo-γ_p (p for parallel). If a similar formalism is applied, **58** forms 11-membered rings across the intramolecular hydrogen bond between amino acids

Scheme 18. Two 1,1′-Ferrocene Peptide Derivatives That Do Not Form Intramolecular Hydrogen Bonds in Solution



i and $i + 3$, and this we denote a pseudo-β_p turn. Because of the approximate C_2 symmetry of this molecule, the same rules apply to both Cp rings and their substituents.

The strength of the hydrogen bonds in CH_2Cl_2 and $CHCl_3$ solutions of **58** was investigated in relation to that of its positively charged cobaltocenium derivative **59**.[123] It appears that the hydrogen bonding interactions for the cobaltocenium derivative **59** are slightly stronger than those for the ferrocene compound **58**. However, the differences are small and sensitive to experimental conditions. Especially the presence of traces of water has a significant influence on the hydrogen bonding properties.

The ^{57}Fe Mössbauer spectroscopic parameters of **50** and **58** were compared to those of ferrocene (**1**),[159] **46**, and **47**.[123] The isomer shifts for these compounds are very similar, whereas the values for the quadrupole splitting (ΔE_Q) were found to vary in the following order: FcH ($\Delta E_Q = 2.42$ mm s^{-1})[138,159] > ferrocenoyl derivatives **46** and **47** ($\Delta E_Q = 2.32$ mm s^{-1}) > 1,1′-disubstituted derivatives **50** and **58** ($\Delta E_Q = 2.27$ mm s^{-1}). It has been observed before that ΔE_Q decreases when a Cp ring of ferrocene is substituted with an electron-withdrawing substituent, and even more when such a substituent is present on either Cp ring.[137]

Hirao and co-workers prepared the compound Fe(C$_5$H$_4$-CO-Ala-Pro-NHPy)$_2$ (**59**) (NHPy is the amide of 2-aminopyridine) and subsequently transformed it into the *trans*-$PdCl_2$ complex **60**, with the pyridine nitrogen atoms coordinated to the Pd atom.[160] X-ray crystal structures for both compounds were reported; the one for **60** is reproduced in Figure 8. Both compounds **59** and **60** adapt the "usual" ordered conformation in the solid state as well as in solution, with the Ala-NH group being involved in intramolecular hydrogen interactions with the Ala-CO moiety of another strand. In fact, coordination of the pyridine rings to the Pd atom stabilizes the intramolecular hydrogen bonds significantly, in solution as well as in the solid state. In another paper, Hirao and Moriuchi prepared and structurally characterized the adduct of **59** with (1*R*,3*S*)-camphoric acid.[161] Recently, Hirao and Moriuchi presented an account on some of their results on ferrocenoylpeptides in the Japanese language.[162]

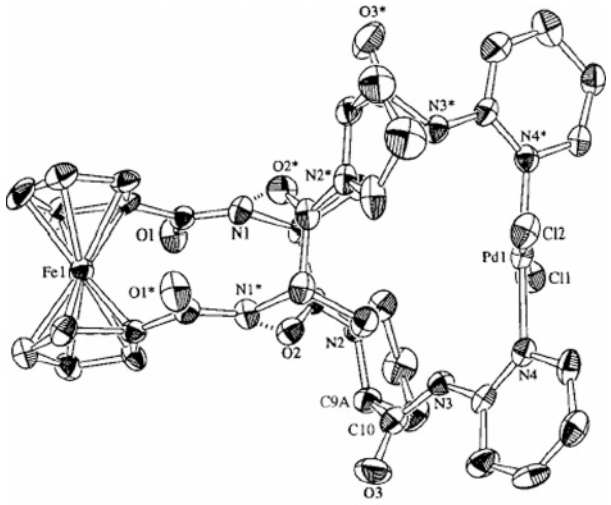
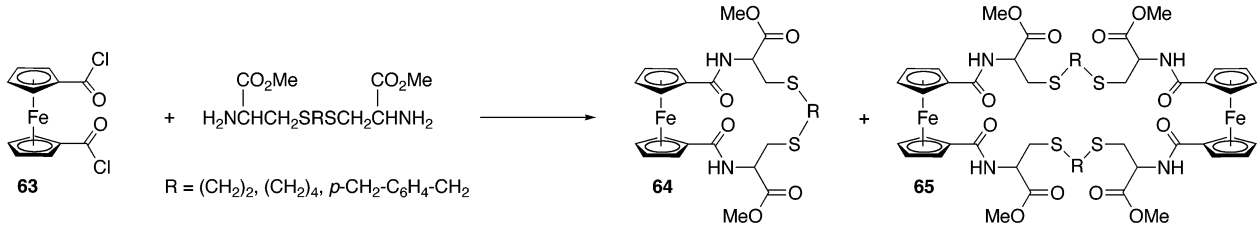


Figure 8. X-ray single-crystal structure for the $PdCl_2$ complex **60**. Reprinted with permission from ref 160. Copyright 2001 The American Chemical Society.

An important point is that an ordered structure will only occur if the terminal amino groups of the amino acids and/or peptides form amides with a carboxylic acid that is *directly* attached to the Cp ring. The groups of Hirao and Beck prepared compounds **61** and **62** with a different longer linker (Scheme 18), and they found that these do not exhibit an intramolecular hydrogen-bonded conformation in solution.[154,163]

Han et al. prepared macrocyclic compounds from ferrocene-1,1′-dicarboxylic acid chloride (**63**) and pseudo-cystine derivatives with various linkers between the sulfur atoms.[164] The synthesis yielded 1,1′-ferrocenophane derivatives **64** as well as cyclodimers **65** (Scheme 19). The redox potential for the Fe(C$_5$H$_4$-

Scheme 19. Synthesis of Cysteine-Bridged Pseudo-1,1′-ferrocenyl Dipeptides

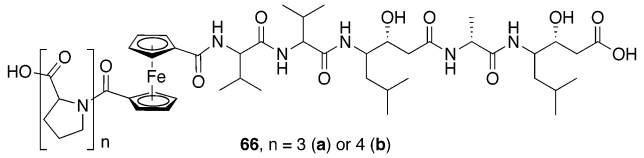


$R)_2/Fe(C_5H_4\text{-}R)_2^+$ couple of the 1,1′-ferrocenophane derivatives **64** is dependent on the nature of the anion. In particular, the addition of fluoride resulted in a pronounced redox potential shift of about 120−150 mV to more positive values. From the occurrence of this shift of the redox potential, it is anticipated that halide ions occupy a position in the binding pocket of the 1,1′-ferrocenophane. The binding is stabilized by hydrogen bonds to the two NH moieties and, in the case of the ferrocenium redox state, by an additional electrostatic interaction between the halide and the iron atom. In a subsequent report,[165] cystine and cystine di- and tripeptides $((AA)_n\text{-}Cys)_2$ (AA = Gly, Ala, Leu, Met, Pro; $n = 0, 1, 2$) were synthesized and reacted with **63** to form ferrocenophanes with a cavity of different size. Into this cavity, alkaline and alkaline earth cations could bind. CD spectrosocpy and cyclic voltammetry were used to determine binding constants. For one derivative, high binding affinity for Mg^{2+} and Ca^{2+}, along with a high preference for Ca^{2+} over K^+, was observed, which was even better than the values for the natural ionophore valinomycin. Recently, other macrocyclic amino acid cystamine derivatives $Fe(C_5H_4\text{-}CO\text{-}AA\text{-}NHC_2H_4S)_2$ were reported (AA = Ala, Val, Leu).[166] These compounds show strong intramolecular hydrogen bonding and structures related to **49**, as discussed above. Their electrochemical properties were also investigated in solution as well as immobilized on Au electrodes.[167]

Recently, Kraatz and co-workers prepared compounds of the general formula $Fe(C_5H_4\text{-}CO\text{-}Pro_n\text{-}OBn)_2$, with $n = 1-4$.[168] As observed for the monosubstituted compounds $Fc\text{-}CO\text{-}Pro_n\text{-}OBn$,[129] the proline chains of the 1,1′-disubstituted compounds display the left-handed helical arrangement characteristic for polyproline II. For the compound $Fe(C_5H_4\text{-}CO\text{-}(Pro)_2\text{-}OBn)_2$, the ordered conformation adapted by the other 1,1′-disubstituted derivatives presented above is not observed, because the Pro−amide linkages lack NH moieties. The redox potential for the $Fe(C_5H_4\text{-}R)_2/Fe(C_5H_4\text{-}R)_2^+$ couple was found to shift to a small extent to less positive values in going from $Fe(C_5H_4\text{-}CO\text{-}Pro\text{-}OBn)_2$ to $Fe(C_5H_4\text{-}CO\text{-}Pro_4\text{-}OBn)_2$, a trend similarly observed for the monosubstituted derivatives $Fc\text{-}CO\text{-}(Pro)_x\text{-}OBn$.[129]

Interestingly, it was reported that the synthesis of $Fe(C_5H_4\text{-}CO\text{-}Pro_n\text{-}OBn)_2$ with $n = 2$, 3, or 4 yielded a second class of compounds, that is, the "incomplete" substituted derivatives $Fe(C_5H_4\text{-}CO\text{-}Pro_n\text{-}OBn)(C_5H_4\text{-}CO\text{-}OBt)$.[168] In a subsequent paper, Kraatz and coworkers reacted the "incomplete substituted" derivatives with $n = 3$ and 4 with a pepstatin analogue to obtain the conjugates **66** shown in Scheme 20.[169]

Scheme 20. Structure of the 1-Oligopropyl-1′-pepstatin Fereocene Derivatives 66



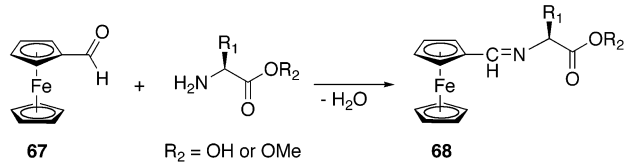
Pepstatin is a potent naturally occurring inhibitor of aspartate proteases, including HIV-1 protease and pepsin.[170−172] Current work to test whether these derivatives have the ability to inhibit aspartate proteases is in progress, according to ref 169.

2.3. Imine and Amine Formation with Ferrocenecarbaldehyde

2.3.1. Imines from Ferrocenecarbaldehyde

Imines from ferrocenecarbaldehyde (**67**) and amino acids or amino acid esters can be prepared in an easy way (**68**, Scheme 21). Various solvents have been

Scheme 21. General Synthesis of Imines 68 from Amino Acids and Ferrocenecarbaldehyde 67



used for this synthetic transformation, such as EtOH,[176] MeOH,[177] or $CHCl_3$.[178] The reactions are performed either at room temperature[177] or at slightly elevated temperatures, ranging from 60 to 80 °C.[177,178] However, one report appeared in the literature in which this type of imines was obtained under solvent-free conditions at room temperature.[179] An overview of the constitution of the reported imines between **67** and amino acids or amino acid esters is given in Table 3.

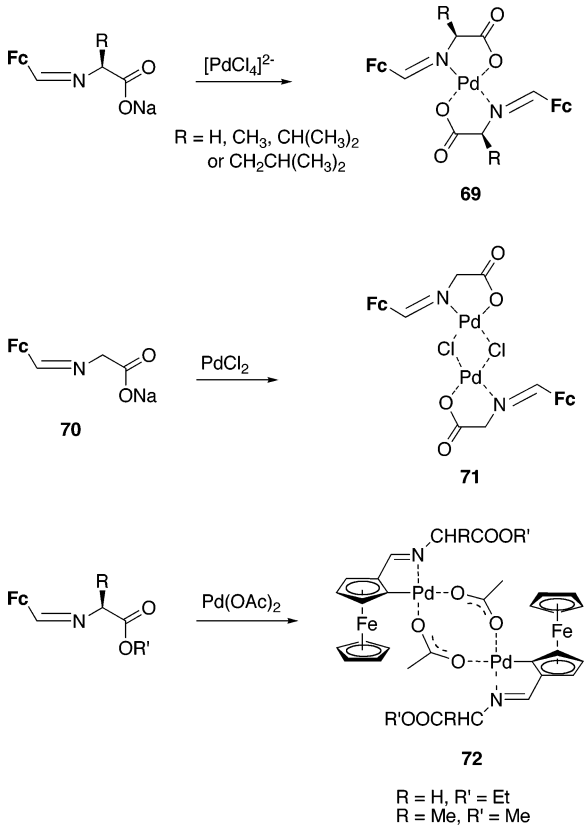
Table 3. Constitution of the Reported Imines 68 from 67 and Amino Acid Derivatives[a]

compound	ref	compound	ref
Fc-C=N(Gly-OH)	176, 177	Fc-C=N(Gly-OEt)	176, 177
Fc-C=N(DL-Ala-OH)	176	Fc-C=N(Ala-OH)	177
Fc-C=N(Ala-OMe)	179	Fc-C=N(DL-Ala-OEt)	176
Fc-C=N(Ala-OEt)	179	Fc-C=N(β-Ala-OH)	176
Fc-C=N(DL-Val-OH)	176	Fc-C=N(Val-OH)	177
Fc-C=N(DL-Val-OEt)	176	Fc-C=N(DL-Leu-OH)	176
Fc-C=N(Leu-OH)	177	Fc-C=N(Leu-OMe)	178
Fc-C=N(Leu-OEt)	176	Fc-C=N(DL-Ile-OH)	176
Fc-C=N(Ile-OMe)	179	Fc-C=N(DL-Asp-OH)	176
Fc-C=N(Glu-OH)	176	Fc-C=N(Phe-OMe)	178
Fc-C=N(DL-Tyr-OH)	176	Fc-C=N(Tyr-OH)	176
Fc-C=N(Met-OH)	176, 178	Fc-C=N(Arg-OH)	176
Fc-C=N(His-OMe)	179	Fc-C=N(Ser-OMe)	179
Fc-C=N(Lys(N=C-Fc)-OMe)	179	[Fc-C=N(Cys(S-)-OH)]$_2$	176

[a] These imines are quite stable compounds in the absence of aqueous acids. They can be reduced with $NaBH_4$ or H_2/Pd to yield the more stable secondary amine derivatives, which will be described in section 2.3.2.

Amino acid ferrocenylimines have been used as ligands for transition metal ions. Beck and coworkers prepared Pd(II) complexes with imines from **67** and the amino acids Ala, Gly, Val, and Leu as ligands.[177] The compostion of the isolated complexes depends on the Pd(II) salt and the type of amino acid derivative, as illustrated in Scheme 22. With Na_2PdCl_4 and imines from **67** and the free carboxylic acid of Ala, Leu, and Val, mononuclear complexes with a ligand/Pd ratio of 2:1 were obtained (**69**). On the other hand, the reaction of $PdCl_2$ with the corresponding ferrocene imine glycine derivative **70** yielded the dinuclear chloro-bridged Pd(II) complex

Scheme 22. Pd Complexes from Ferrocene Imino Acids



71. The reaction of $Pd(OAc)_2$ with the imines between **67** and H-Gly-OMe and H−Ala-OMe resulted in isolation of acetato-bridged species **72**, in which ortho-palladation of the ferrocenyl-Cp ring has occurred. Several of these complexes, including an ortho-palladated derivative, have been structurally characterized by X-ray crystallography. In summary, two types of chelating modes have been observed, the normal N, O coordination mode (**69** and **71**) and also the unusual C, N bidentate mode (**72**).

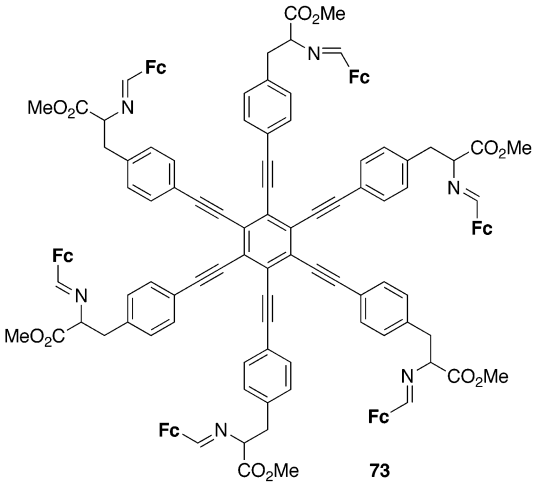
In a later paper, Beck and co-workers prepared imines from tri-, tetra-, and hexasubstituted benzenes with *p*-ethynylphenylalanine methyl ester.[180] As a representative example, the molecular structure of the hexaferrocene derivative **73** is depicted in Scheme 23.

The same 21-mer peptide, which was derivatized with ferrocenecarboxylic acid (**40**) (see section 2.2.1), was also reacted with ferrocenecarbaldehyde (**67**). Also in this case, the six lysine side chain NH_2 groups were labeled and the resulting conjugate was found to adapt a similar helical conformation.[141,142]

2.3.2. Ferrocenylmethylamines via Reduction of the Imines

Ferrocenylmethylamine derivatives **74** have been prepared via reduction of the imines from ferrocenecarbaldehyde (**67**) and amino acids with $NaBH_4$ or H_2/Pd. Alternatively, these amines can be synthesized directly in a one-pot procedure from **67**, the amino acid derivative, and the appropriate reducing reagent (Scheme 24). Up to now, a considerable number of Fem derivatives have been reported, as

Scheme 23. Structure of the "Star-Shaped" Hexakis(ferrocene imino acid) Benzene Derivative 73



Scheme 24. Formation of Ferrocenylmethyl (Fem) Amino Acids 74

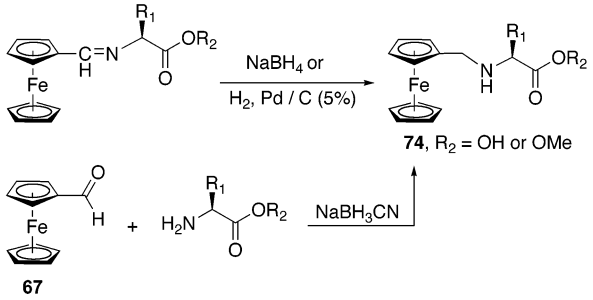


Table 4. Overview of the Reported Fem Amino Acid Derivatives

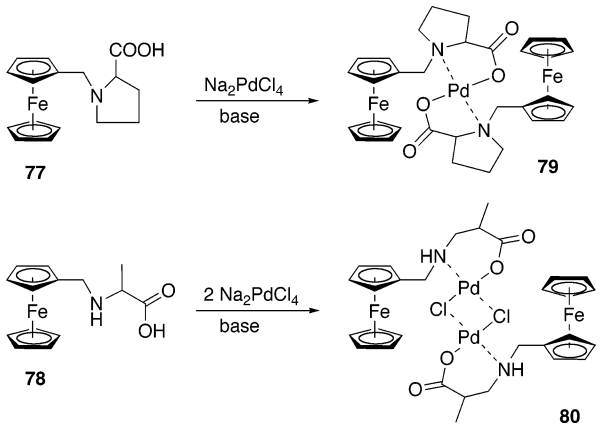
compound	ref	compound	ref
Fem-Gly-OH (**75a**)	182	Fem-Gly-OMe (**75b**)	182
Fem-Ala-OH (**78**)	177, 182	Fem-Ala-OMe	182
Fem-Pro-OH (**77**)	177	Fem-Met-OMe	178
Fem-Phe-OMe	178	Fem-Phe-OH (**84**)	178, 182
Fem-Phe-O^{t}Bu	182	Fem-Leu-O^{t}Bu	182
Fem-Leu-OMe	178	Fem-Val-O^{t}Bu	178
Fem_2-Gly-OMe (**76**)	181		

shown in Table 4. Some of these compounds, in particular the glycine derivative Fem-Gly-OH (**75a**, Fem = ferrocenylmethyl), found application in peptide synthesis as lipophilic amino acid residues, with masked amide moieties. This is treated in section 2.5.1. In a stoichiometric reaction, small amounts of the bimetallic compound $(Fem)_2$Gly-OMe (**76**) were isolated in addition to the expected mono-Fem derivative Fem-Gly-OMe (**75b**).[181]

Beck and co-workers used Fem-Pro-OH (**77**) and Fem-Ala-OH (**78**) as ligands for Pd(II).[177] The reaction of Na_2PdCl_4 with **77** and **78** yielded two different types of complexes (Scheme 25). With **77**, the trimetallic (1Pd and 2Fe) compound **79** with a ligand versus Pd ratio of 2:1 was obtained. Under identical conditions, the synthesis with **78** resulted in isolation of the dimeric bis-μ-chloro Pd(II) complex **80**.

Metzler-Nolte and co-workers explored the peptide-coupling chemistry of Fem-amino acid derivatives. Coupling of amino acids and peptides to the C-

Scheme 25. Pd Complexes from Ferrocenylmethyl Amino Acids



terminus of Fem-amino acid esters proceeds readily after hydrolysis of the ester functionality and subsequent HBTU activation.[178,183,184] In contrast, HBTU activated N-protected amino acids showed no reactivity toward the secondary amino group of Fem-Phe-OMe (Scheme 26). This can probably be attributed to steric effects, because Fem-benzylamine (**81a**) and Fem-methylbenzylamine (**81b**) react smoothly with HBTU-activated amino acids to yield derivatives **82a** and **82b**, respectively.[178,183,185] L-Leu, as well as L- and D-Ala, was successfully used for this coupling reaction. NMR investigations in combination with molecular modeling were used to elucidate the structure of these conjugates in solution and explain the differences in reactivity.[178]

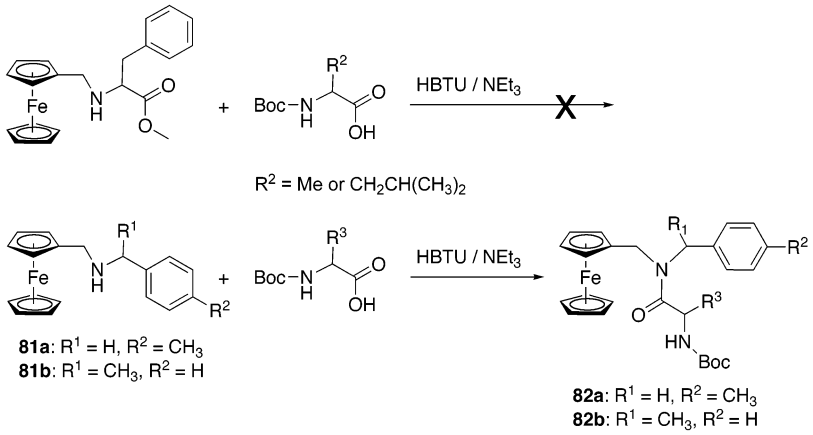
These ferrocenylbenzylamine derivatives can be applied as an anchoring group for the C-terminus of amino acids and peptides.[185] In that respect, the labeling is complementary to **40**, which can serve as a marker for the N-terminus of peptides and amino acids. Starting from the Fem dipeptide derivative **83**, various interesting bimetallic derivatives such as **85** and **86** have been prepared in the Metzler-Nolte group as well (Scheme 27).[178,183]

2.4. Other Attachment Methods

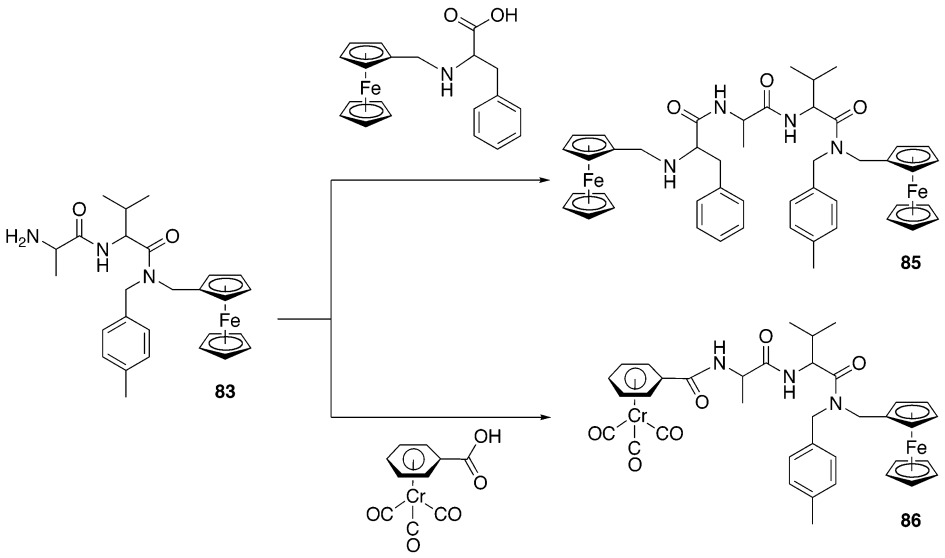
2.4.1. Sonogashira Coupling

The Sonogashira reaction is a Pd-catalyzed cross-coupling of a terminal alkyne with a halogen-substituted aromatic ring, according to Scheme 28.[186−188] Although a number of variations have been published recently, including Cu-free examples,

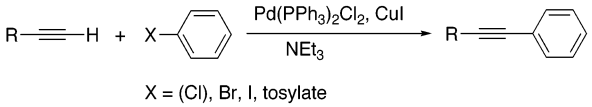
Scheme 26. Difference in Reactivity of the Secondary Amino Group of Fem Amino Acid Esters and Amines toward Amino Acids



Scheme 27. Bimetallic Ferrocenyl Amino Acid Derivatives



Scheme 28. Sonogashira Coupling



the most common catalyst system is still the combination of $PdCl_2(PPh_3)_2$ and CuI. The reaction requires anaerobic conditions, but solvent mixtures containing up to a few percent of water are tolerated, thus making the use of commercial undried solvents possible. The Metzler-Nolte group has developed a practical and flexible method for labeling the C- or N-terminus of amino acids and peptides, consisting of a two-step procedure.[189−191] Two similar synthetic routes to attach a marker to the C-terminus have been explored, as shown in Scheme 29.

The first step for introducing a label on the C-terminus consists of functionalizing a Boc-protected amino acid or Boc-protected dipeptide at its C-terminus by amide formation with propargylamines **87** (R = H (**a**), Et (**b**)) or with *p*-iodoaniline (**88**). The synthesis starts with the preparation of amides **89** and **90** from ferrocenecarboxylic acid (**40**) and **88** or **87**, respectively. These amides are prepared via standard solution-phase peptide synthesis methods, by employing isobutylchloroformiate and *N*-methylmorpholine.[192] In the second step, the Sonogashira coupling is performed, yielding the ferrocene-functionalized amino acids and peptides such as **91** and **92**. The ferrocene diethylpropargylamide derivative **90b** proved to be superior to the ferrocene propargylamide congener **90a** because of its significantly enhanced solubility and more facile purification of conjugates such as **92b**.

This method is not restricted to C-terminus labeling, but via a slight modification it can be adapted for the introduction of a marker on the N-terminus, as shown in Scheme 30. The amide of *p*-iodobenzoic acid and H-Leu-OMe was reacted with **90b** under identical Sonogashira coupling conditions, yielding the N-terminally labeled compound **93**.[190]

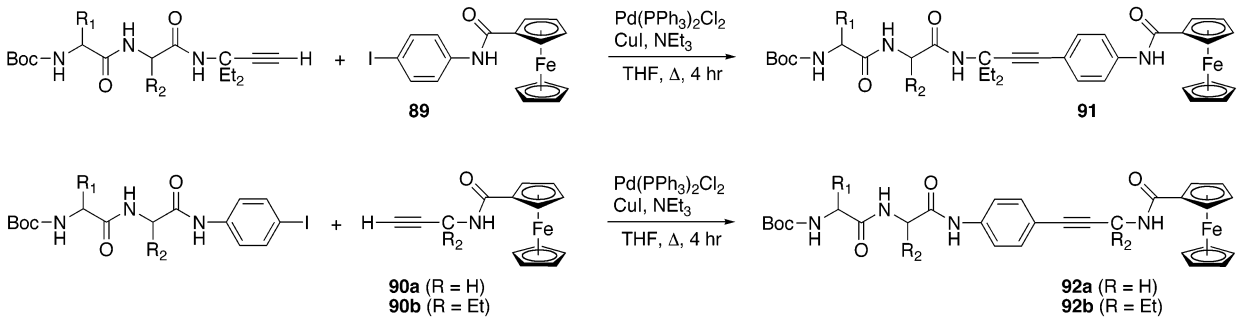
This two-step labeling procedure is very attractive for several reasons. First of all, both steps, amide formation and Sonogashira coupling, proceed in good yields, ranging from 70 to 95%. Second, commercial reagent-grade solvents can be employed for either step. Only for the Sonogashira coupling step is the use of deoxygenated solvents required. Third, the method is versatile and of wide scope, as it tolerates a variety of functional groups, such as alcohols (Ser), thioethers (Met), esters, and amides.[191]

2.4.2. Other Methods for Labeling the C-Terminus

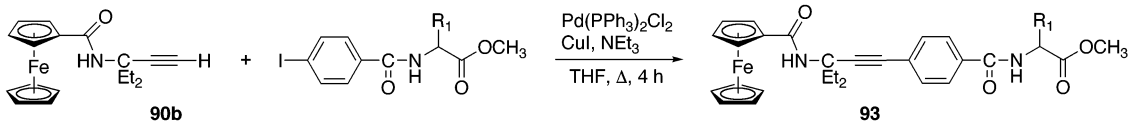
In addition to the Sonogashira procedure, several other methods for the derivatization of the C-terminus have been developed. One of these, amide formation between Fem-benzylamine or Fem-methylbenzylamine and the C-terminus carboxylic acid, has already been presented in section 2.3.2. Other methods include ester or anhydride formation, and examples will be given below.

Eisenthal and co-workers prepared two ferrocenoyl derivatives **94** of the channel-forming peptide alamethicin (ALM).[193] One of the derivatives (**94a**) was prepared from **40**, whereas the other (**94b**) was synthesized from ferrocene-1,1′-dicarboxylic acid. Therefore, the compounds differ in the unconjugated Cp ring; **94a** has a Cp ring, whereas **94b** has a η-C_5H_4-COOH moiety (Scheme 31). In both cases, the ferrocenoyl moieties were linked via ester formation to the C-terminus of the 20-mer peptide in solution. Alamethicin is a channel forming peptide, produced by the fungus *Trichoderma viride*, and belongs to the class of peptaibols. Alamethicin self-associates in lipid bilayers, in this way forming voltage-dependent

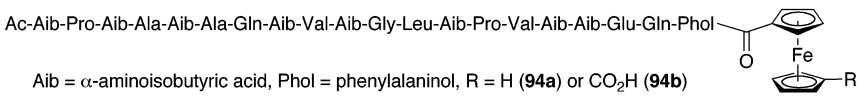
Scheme 29. Two Methods for Labeling the C-Terminus of Peptides by Sonogashira Coupling



Scheme 30. Scheme for Labeling the N-Terminus of Amino Acids and Peptides by Sonogashira Coupling



Scheme 31. Structures of Two Alamethicin Ferrocene Derivatives 94[a]



[a] Note that the ferrocenoyl group is bound to the C-terminus of the peptide.

ion channels.[194−197]

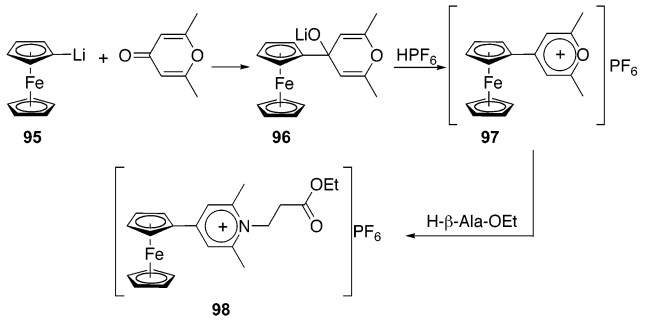
In the reduced (neutral) form, both ferrocenoyl alamethicin derivatives **94** are shown to form voltage-dependent channels in planar lipid bilayers at positive potentials, with conductance properties similar to unmodified alamethicin. While in the lipid bilayer form, oxidation of **94b** results in a shorter lived channel whereas the oxidation of **94a** causes a time-dependent elimination of channel openings, which can be restored by increasing the trans-bilayer potential. Pretreatment of the ferrocenoyl peptides with oxidizing agents alters their single-channel properties in a qualitatively similar manner.

Although the following example is not strictly a labeling method for the C-terminus, it is presented at this stage because attachment of the marker resembles the previous example. Toniolo, Maggini and co-workers prepared a nonapeptide with a 3_{10}-helical conformation, in which two tyrosine phenol rings were esterified with ferrocenecarboxylic acid.[198−200] These synthetic helical peptides were shown to have a hydrophobic binding cavity, which can serve as an efficient host for [60] fullerene.

2.4.3. Other Methods for Labeling the N-Terminus

An unconventional procedure for labeling the N-terminus of amino acids has been developed by Jaouen and co-workers.[201] The method consists of the synthesis of a pyrilium salt from lithioferrocene (**95**) and 2,6-dimethyl-γ-pyrone (**96**) and subsequent acidification (Scheme 32). This pyrilium salt **97** reacts

Scheme 32. Reaction of a Ferrocenyl Pyrilium Salt with β-Alanine



with amines, in this case β-alanine, to yield pyridinium salt **98**. To the best of our knowledge, this method has not been adapted for the introduction of a ferrocene tag to larger biomolecules.

Gallagher et al. prepared the benzoic acid derivative p-Fc-C_6H_4-CO_2H and coupled it to alanine via amide formation.[202] This method is related to derivatization with ferrocene carboxylic acid, treated in section 2.2.1.

In two papers and a patent, Eckert and Koller described a variety of ferrocene compounds for derivatization of the N-terminus of amino acids and the lysine-NH_2 moieties of the protein BSA.[146,203,204] The objective was to investigate which of the reagents was best suited for labeling purposes and subsequent electrochemical detection of the conjugates by HPLC-ECD (high performance liquid chromatography with electrochemical detection). Among the naturally occurring amino acids, only methionine, tyrosine, and tryptophane show a little electrochemical activity, but their redox potential is too high for good selectivity ($> +0.5$ V vs FcH/FcH^+).[205−210] Introduction of a ferrocene moiety will lower the redox potential for detection by at least a few hundred millivolts, the exact value depending on the functionality that is directly attached to the Cp ring. The decrease of the redox potential will result in a substantially increased selectivity.

Reagents for HPLC-ECD derivatization that were investigated by Eckert and Koller include, for example, Fc-SO_2-Cl (**99**), Fc-CO-Cl (**41**), ferrocenecarboxylic acid anhydride (**100**), ferrocenepropionic acid anhydride (**101**), and ferrocenylmethyl isocyanate (**102**). Of these derivatives, **101** was found to be best suited on the basis of labeling results and the sensitivity for electrochemical detection of the corresponding conjugates.[203] However, some results in the literature are contradictive, because Koppang et al. found **99** and **41** to be good derivatization reagents for HPLC-ECD,[148] whereas Eckert and Koller found these to be unsuitable.[203] In a related paper, Shimada et al. compared ferrocenyl isothiocyanate and ferrocenylethyl isothiocyanate for glycine and 4-aminobutyric acid labeling.[211] The latter displayed higher reactivity and more favorable electrochemical properties and was adapted for 4-aminobutyric acid determination in biological samples by HPLC-ECD.

2.4.4. Labels for the Thiol Group in Cysteine

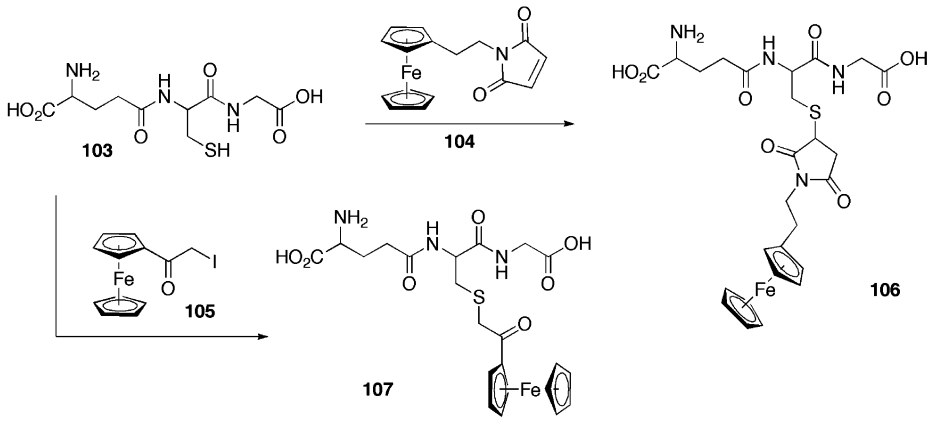
The labeling methods treated thus far in this review are suitable for the derivatization of amino and carboxylic acid moieties of peptides and amino acids. However, selective labels for the sulfhydryl group of cysteine have also been reported.[212−214] For the labeling of glutathione **103**, both ferrocenylethyl maleimide (**104**) and ferrocenyl iodoacetamide (**105**) (Scheme 33) turned out to be very effective to yield **106** and **107**, respectively.[212,214] A related maleimide derivative has also been used to label the hexapeptide Ac-Arg-Arg-Ala-Ser-Leu-Cys-OH.[213] This labeled hexapeptide was applied for the detection of serine phosphorylation by protein kinase A via electrochemical methods. The use of **104** for the labeling of Cys residues in proteins such as cytochrome P450 is described in section 3.1.3. Ferrocenyl iodoacetamide (**105**) has also been used for the labeling of BSA (seee section 3.2.2) and a 5′-thiolated oligonucleotide (see also section 4).[214]

It should be noted that the reagents Fc-HgCl (**108**) and ferrocenoylchloro acetamide (**109**) (Fc-CO-NH-CH_2Cl) have been used for the introduction of a ferrocene-based marker on Cys-SH groups in proteins. Relevant examples will be presented in section 3. In section 2.5.1, the use of the ferrocenylmethyl rest as a protecting group for the cysteine SH moiety during peptide synthesis is presented.

2.4.5. Miscellaneous

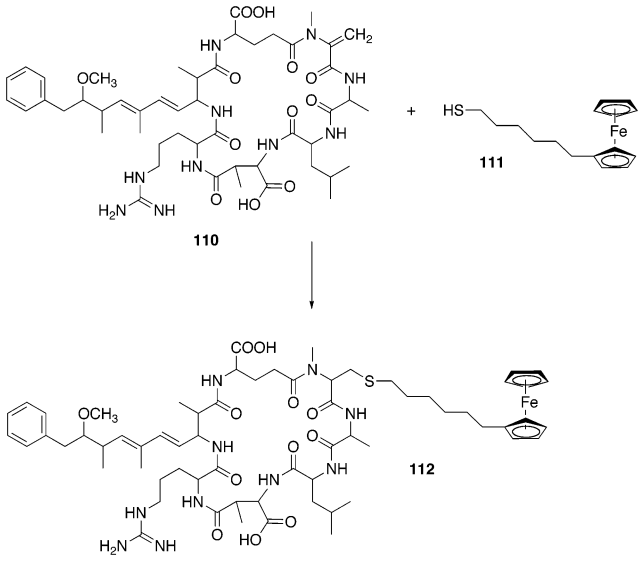
Microcystins are a class of low-molecular-weight cyclic peptide hepatoxins. These microcystins are produced by cyanobacteria (blue-green algae) in eutropic lakes and drinking water reservoirs, par-

Scheme 33. Two Ferrocene Reagents for Labeling Glutathione 103



ticularly in global regions that are hot and relatively dry, such as China, Australia, and South Africa.[215] Because these substances are hazardous to human beings, cattle, and wildlife, it is important to develop methods for rapid detection of these toxins. Lo, Lam, and co-workers very recently developed an elegant method that makes the detection of a particular microcystin, namely microcystin-LR, possible via a quick electrochemical measurement.[216] This method is based on the specific derivatization of the exocyclic double bond of the α,β-unsaturated carbonyl moiety of microcystin-LR (**110**) with ferrocenylhexanethiol (**111**) (Scheme 34) and the subsequent electrochemical detection of the Fc/Fc^+ moiety in the ferrocene-tagged conjugate **112**. The limit of detection was found to be ~18 ng, which is in the range of other common detection methods, such as thin-layer chromatography and HPLC with UV detection. It must be noted that much lower levels of microcystins can be detected with more expensive methods, such as fluorescence spectroscopy and HPLC-MS. The electrochemical method, however, is quick, specific, and inexpensive.

Scheme 34. Derivatization of Microcystin 110 with 6-Ferrocenylhexanethiol 111

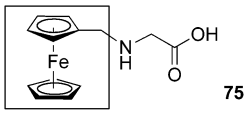


2.5. Application of Ferrocene Compounds in Peptide Synthesis

2.5.1. The Ferrocenylmethyl (Fem) Group as a Protecting Group

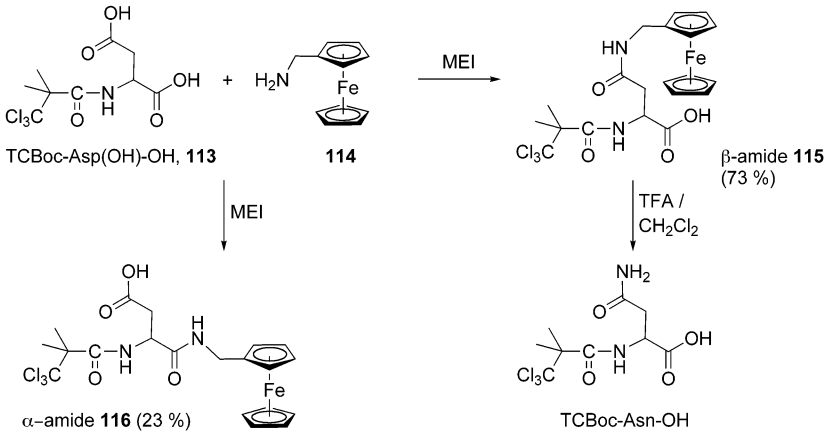
Eckert and co-workers introduced the ferrocenylmethyl (Fem) group as a lipophilic rest to mask the highly polar glycine amide moiety during peptide synthesis in solution.[181,182,217] This Fem moiety (Scheme 35) can be introduced on glycine via imine formation with ferrocenecarbaldehyde, followed by reduction to yield **75** (see section 2.3). Peptide coupling of this modified glycine residue to other amino acids or peptide fragments proceeds readily by employing the standard carbodiimide method used for peptide synthesis in solution.[218,219] The advantages of employing this masked glycine derivative for peptide synthesis in solution are as follows: (1) The intermediates and products show increased solubility in apolar organic solvents as a consequence of the lipophilic Fem rest. This results in complete reactions and improved yields. In addition, the chromatographic purification over silica is facilitated, since the products can be eluted with ethyl acetate/hexane rather than with more polar $MeOH/CHCl_3$ mixtures. (2) The ferrocene chromophore of the intermediates and products facilitates product identification and isolation during chromatographic purification. (3) The growing peptide is stable against racemization during the coupling steps, when it is assembled in stepwise fashion starting from the C-terminus. Cleavage of the Fem group is postponed until the desired amino acid sequence has been obtained. Removal of the Fem group is accomplished by treatment with TFA/β-thionaphthol in CH_2Cl_2 at room temperature for 2−4 h.[181,182,217] Under these conditions, Boc and *tert*-butyl protecting groups are simultaneously cleaved. The usefulness of Fem-Gly derivatives for peptide synthesis in solution is illustrated by the successful synthesis of [Leu^5]-enkephalin and H-$(Gly)_6$-OH.[181]

Scheme 35. The Ferrocenylmethyl (Fem) Group 75

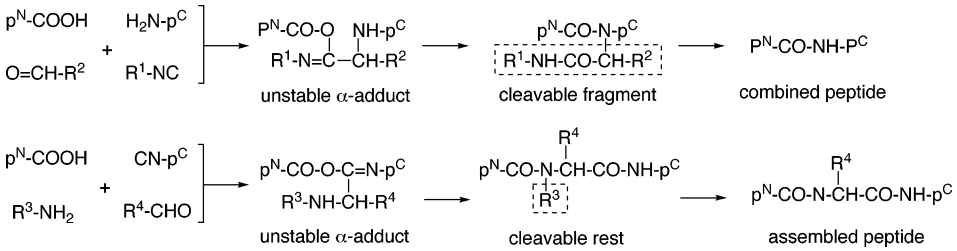


Ferrocenylmethyl (Fem)

Scheme 36. Adaption of the Fem Group for the Preparation of Masked Asparagine Residues



Scheme 37. Two Types of Four-Component Coupling (4-CC) Reactions



The Fem group can also be adapted for the synthesis of masked asparagine residues from aspartic acid. The strategy of this elegant method is outlined in Scheme 36. TCBoc-protected aspartic acid **113** is reacted with ferrocenylmethylamine (**114**) in DMF in the presence of 2-morpholinoethyl isocyanide (MEI).[217] This yields 73% of the desired β-isomer **115**, together with 23% of the α-isomer **116** as a by-product, after purification by column chromatography. The β-isomer **115** can be used as an Asn building block in peptide synthesis. Similar to the Fem-Gly derivative, the reaction of the β-isomer with TFA/β-thionaphthol in CH_2Cl_2 liberates the asparagine. The usefulness of this Asn-protected residue in combination with the Fem-Gly derivative was demonstrated for the synthesis of the 24−31 octapeptide sequence of human-β-endorphin.[217]

The Fem rest has also been used as a protecting group for the cysteine thiol functionality.[220,221] The compound H-Cys(S-Fem)-OH can be conveniently prepared by reacting cysteine with ferrocenylmethanol in aqueous acetone in the presence of a catalytic amount of acid. This side-chain-protected cysteine can be transformed into the N-terminus Boc- or Fmoc-protected derivatives. These, in turn, are useful building blocks for peptide synthesis, as demonstrated by the synthesis of glutathione **103**. After complete assembly of the peptide, the ferrocenylmethyl moiety can be cleaved by TFA or by soft heavy metal ions, such as Ag^+ or Hg^{2+}.[220,221]

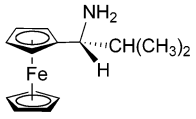
2.5.2. Peptide Synthesis via Four-Component Reactions with Ferrocene Alkylamines

The four component condensation (4-CC) of isocyanides (also called isonitriles), discovered by Ugi in the late 1950s,[222] provides an alternative to the classical methods for the synthesis of peptides in solution.[223−228] The conventional methods are characterized by the reaction of a C-terminally protected amino acid or peptide with an N-terminally protected amino acid or peptide in the presence of a coupling reagent, such as isobutylchloroformiate or a carbodiimide derivative.[218,219,229]

The four-component condensations proceed in a different way, and can be subdivided into two categories, according to Scheme 37.[230−235] In the first method, two peptide fragments, one of them having an unprotected carboxylic acid group and the other having a free amino moiety, are reacted with an isocyanide and an aldehyde. This leads to formation of an unstable α-adduct, which spontaneously rearranges to form a tertiary amide. The groups R^1 and R^2 are usually chosen such that the fragment R^1-NH-CO-CH-R^2 is easily cleaved under acidolytic conditions. The result in this case is the coupling of the two peptide or amino acid fragments (Scheme 37, top).

In the other method, an N-protected peptide or amino acid, a (chiral) primary amine, an aldehyde, and an isocyanide−peptide fragment are combined to yield, also in this case via spontaneous rearrangement of an unstable α-adduct, a compound in which a new peptide segment has formed (Scheme 37, bottom). When ammonia or achiral primary amines are used, the newly formed amino acid residue is either achiral or racemic.[236−240] If the newly formed peptide segment should correspond to a chiral amino acid, it is necessary to use a chiral primary amine as a chiral inducer. Chiral α-ferrocenylalkylamines, in particular compound **117** shown in Scheme 38, have found widespread application in these stereoselective four-component condensations.[241−248] This is because (1) they were shown to have strong asymmetric inducing power and (2) they were cleaved

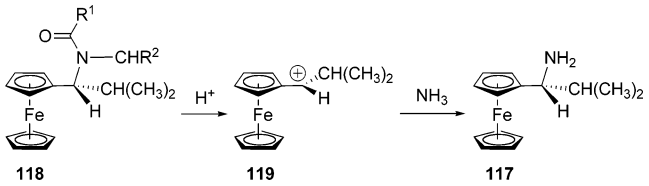
Scheme 38. Particularly Powerful Ferrocene-Based Chiral Inducer (117) for Stereoselective 4-CC Reactions



(*R*)-1-ferrocenyl-2-methyl-propanamine **117**

easily under acidolytic conditions. In fact, the chiral primary amine **117** can be regenerated from the peptide **118** by a series of steps, as shown in Scheme 39.[245,249,250] No racemization occurs because the rotational barrier of the cation **119** is high and the nucleophile (NH_3) can only attack the cation from the upper side of the ferrocene due to steric reasons.

Scheme 39. Cleavage of the Ferrocenylalkyl Fragment and Regeneration of the Chiral Amine 117

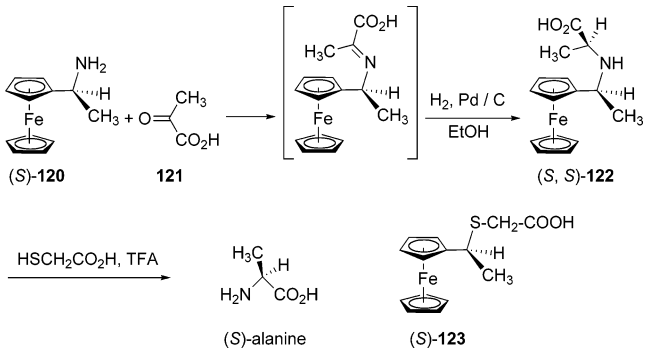


118 **119** **117**

As a proof of concept, stereoselective 4-CC employing chiral ferrocenylalkylamines has been used to synthesize tetravaline and glutathione **103** of high enantiomeric purity.[243,244,248] In two related patents, it was reported that stereoselective 3-CC with isobutyraldehyde-*N*-α-ferrocenylethylamine as one of the components yields *N*-acyl-α-amino acid amides and peptides after TFA cleavage.[251,252] These compounds were claimed to be useful as paint and varnish thickeners and as plastisizers for poly(vinyl chloride).

Ratajczyak and Czech used a chiral ferrocenylamine for stereoselective alanine synthesis, as shown in Scheme 40. The chiral amine **120** was reacted with pyruvic acid (**121**) followed by hydrogenation in situ to yield **122**, and in the next step, the amino acid was cleaved from the ferrocenylamine with thioglycolic acid.[253] The thereby formed ferrocenylthioglycolic acid (**123**) can be transformed into the *S*-configured amine **120** by reaction with $HgCl_2$ in aqueous NH_3, a procedure previously published by Ratajczak.[249,250] Depending on the chirality of the amine, either D- or L-alanine is obtained with an enantiomeric excess ranging from 53 to 60%.

Scheme 40. Synthesis of *S*-Alanine from Pyruvic Acid and (*S*)-Ferrocenylethylamine (120)



(*S*)-**120** **121** (*S*, *S*)-**122**

(*S*)-alanine (*S*)-**123**

3. Conjugates of Ferrocene with Proteins

Salmain and Jaouen recently published a review article on the side-chain-selective and covalent labeling of proteins with transition organometallic complexes.[47] Their article focuses on organometallic labels different from ferrocene and their side chain selectivity.

3.1. Redox Proteins

3.1.1. Glucose Oxidase (GOD)

Glucose oxidase (GOD) from *Aspergillus niger* is a 150−180 kD dimeric glycoprotein, with the carbohydrate part of the enzyme constituting about 16−24% of the molecular weight.[254,255] Furthermore, the enzyme contains two tightly bound FAD cofactors, which are essential for activity.[256] Dissociation of the two subunits only occurs under strongly denaturing conditions (SDS) and is accompanied by the loss of the FAD cofactors.[257] The primary amino acid sequence for the 583 amino acid protein has been determined from the cloned *Aspergillus niger* gene,[258,259] and the crystal structure of the partially deglycosylated enzyme has been resolved to 2.3 Å resolution.[260,261] The enzyme contains 32 reactive NH_2 groups, that is 15 lysine residues per monomer and a N-terminal amino group.[258,259,262]

Glucose oxidase oxidizes β-D-glucose to δ-D-glucolactone via a two-electron process (see eq 1). The FAD cofactor serves as the electron donor and is reduced to $FADH_2$. In a next step, $FADH_2$ is regenerated by oxidation with dioxygen, which leads to H_2O_2 formation (eq 2).

$$\text{GOD(FAD)} + \beta\text{-D-glucose} \rightarrow \text{GOD(FADH}_2\text{)} + \delta\text{-D-glucolactone} \quad (1)$$

$$\text{GOD(FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOD(FAD)} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{GOD(FADH}_2\text{)} + 2\text{Fc-R}^+ \rightarrow \text{GOD(FAD)} + 2\text{Fc-R} + 2\text{H}^+ \quad (3)$$

Monitoring of the glucose level in blood is important for people suffering from diabetes. Therefore, there is a need for reliable and quick determination of glucose in solution. The development of such sensor devices has recently been reviewed by Wang.[68] The groups of Hill, Heller, and Bartlett independently reported on the labeling of glucose oxidase with ferrocene derivatives in 1986 and 1987.[263−265] Several additional reports by the groups of Bartlett,[266] Heller,[267−270] and others[271−274] followed in the next decade. The compounds ferrocenecarboxylic acid (**40**) and ferrocenylacetic acid (**124**) were mainly employed as labeling reagents, in combination with the activating reagent EDC. The coupling reactions were performed under various conditions, resulting in conjugates with varying Fc-to-GOD ratios; an overview is given in Table 5. The labeling results in the literature are somewhat contradictive, because derivatization

Table 5. Overview of GOD Derivatization with Various Ferrocene Derivatives

entry number	ferrocene derivative	coupling reagent	denaturing conditions	Fc/GOD ratio	ref
1	Fc-COOH (**40**)	IBCF	none	8	263
2	Fc-COOH (**40**)	EDC	none	8.5	274
3	Fc-COOH (**40**)	EDC	3M urea	12 ± 1	265, 267, 268, 270
4	Fc-COOH (**40**)	EDC	3M urea	13	264, 266
5	Fc-COOH (**40**)	EDC	3M urea	3.6, 8.5, 13, 29 ± 3[a]	272
6	Fc-COOH (**40**)	EDC	3M urea	18.2	274
7	Fc-COOH (**40**)	EDC	3M urea	9.2	278, 279
8	Fc-COOH (**40**)	EDC	400 MPa	7.4	274
9	Fc-COOH (**40**)	EDC/HOSu	none	5 ± 1, 12[a]	272
10	Fc-COOH (**40**)	EDC/HOSu	none[b]	5	271
11	$Fe(C_5H_4\text{-}COOH)_2$	EDC/HOSu	none	7.5	272
12	$Fc\text{-}CH_2\text{-}COOH$ (**124**)	EDC	3M urea	13 ± 1	267, 268, 270
13	$Fc\text{-}CH_2\text{-}COOH$ (**124**)	EDC	3M urea	22	264, 266
14	$Fc\text{-}CH_2\text{-}COOH$ (**124**)	EDC/HOSu	none	10	272
15	$Fc\text{-}(CH_2)_3\text{-}COOH$	EDC	3M urea	29	264
16	$Fc\text{-}CH_2$-caproic acid[c]	EDC/HOSu	none	21	273, 284
17	$Fc\text{-}CH_2$-caproic acid[c]	EDC/HOSu	none	24	285, 286, 287
18	Fc-CHO (**67**)	$NaBH_4$[d]	none	4.2	274
19	Fc-CHO (**67**)	$NaBH_4$[d]	3M urea	8.1	274
20	Fc-CHO (**67**)	$NaBH_4$[d]	none	27.5	274

[a] Various concentrations of the ferrocene carboxylic acids resulted in conjugates with different Fc/GOD ratios. [b] Performed in inverse micelles of the protein in a H_2O/MeCN ratio of 1:75. [c] $Fc\text{-}CH_2\text{-}NH\text{-}(CH_2)_5\text{-}COOH$. [d] $NaBH_4$ is not a coupling reagent but a reducing reagent to transform the labile imine into a secondary amine.

under apparently identical conditions, but performed by different groups, leads to conjugates with different Fc-to-GOD ratio. This is exemplified by mutually comparing entries 3−7 as well as entries 12 and 13 in Table 5. In addition, the derivatization of GOD with ferrocenecaproic acid by Willner, Katz, and co-workers lead to conjugates with ferrocene: protein ratio of 21 or 24 (entries 16 and 17). In other reports the ferrocene/protein ratio was not given.[69,275]

The objective behind the derivatization was to make direct electrochemical communication possible between glucose oxidase and the electrode. The redox center of the enzyme, along with the FAD cofactor, is buried deeply in the core of the enzyme, with the surrounding part of the protein serving as a thick electrically insulating shell. By binding ferrocenyl residues covalently to the enzyme, electron transfer between the FAD cofactor and the electrode can take place via the ferrocene moieties. In fact, (substituted) ferrocenium ions $Fc\text{-}R^+$, which form by oxidation of the ferrocene group, can accept electrons from $FADH_2$ and serve as a substitute for dioxygen (eq 3). Unlike dioxygen, ferrocene is only undergoing a one-electron transfer; regeneration of FAD from $FADH_2$ is thus a stepwise process.

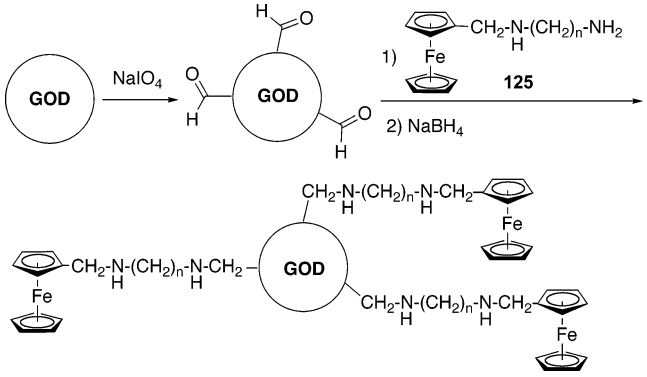
For the derivatization, it was found that denaturing the protein in the presence of 3 M urea resulted in a significant loss of activity of the reconstituted enzyme, even when no ferrocenyl carboxylic acid derivative was added.[271,272] More suitable conditions for obtaining conjugates with higher enzyme activity comprise activation of the carboxylic acid groups of the labeling reagent with EDC and HOSu, without addition of urea. Labeling of GOD with ferrocenylacetic acid (**124**) turned out to be more favorable with respect to derivatization with ferrocenecarboxylic acid (**40**), because the resulting conjugates exhibited higher enzyme activity.

However, for the construction of reagent-less glucose sensors, these conjugates with good enzyme activity did not exhibit satisfying properties. First of all, repeated electrochemical cycles were shown to bring about a gradual decrease in the peak current, which can be interpreted by slow decomposition of the covalently attached redox centers. The second unsatisfying fact was revealed by the work by Mikkelsen, English, and co-workers, who determined the rates for intramolecular electron transfer for some of the conjugates listed in Table 5.[272] Despite the difference in the number of attached ferrocene moieties, the values for electron transfer between the conjugates and the electrode span a narrow range between 0.16 and 0.90 s^{-1}. This suggests that the location of the ferrocenyl groups rather than the number of bound ferrocenyl moieties is rate-determining,[272] in accordance with the Marcus theory of electron transfer.[276,277] An analysis of the—at that time recently determined—crystal structure of GOD showed that the separations between the lysines and the FAD cofactor were all >23 Å.[261] These two drawbacks make clear that an alternative approach is necessary towards electrochemically wired GOD derivatives with excellent mediation between the FAD cofactor and the electrode.

Heller and co-workers tried to improve the electronic communication between the FAD cofactor and the electrode by using ferrocenyl derivatives with a long alkyl chain as labeling reagents, and in addition, these were attached to the carbohydrate part instead of the protein part of the enzyme.[269] In the first step, GOD was oxidized with $NaIO_4$ to yield an average of 6.4 aldehyde groups on the outer surface of the enzyme. Subsequently, aminoferrocenyl residues **125** with differing spacer lengths were tethered to the aldehyde groups via imine formation and subsequent $NaBH_4$ reduction (Scheme 41). By coulometric analysis, the number of introduced ferrocene moieties was estimated to be around 1 for each derivative.

The length of the spacer turned out to play a crucial role for the mechanism of electric communication between the enzyme and the electrode. When attached to longer chains of more than 10 bonds, the

Scheme 41. Modification of Glucose Oxidase on the Peripheral Carbohydrate Part of the Enzyme



ferrocenyl moiety can penetrate the enzyme to a sufficient depth to allow fast intramolecular electron transfer between the flavin centers and the electrode. When attached to short chains of less than 5 bonds, on the other hand, the electron-transfer process was primarily intermolecular, with another modified GOD molecule acting as a conventional diffusing mediator (see Figure 9).[269] The results obtained with short

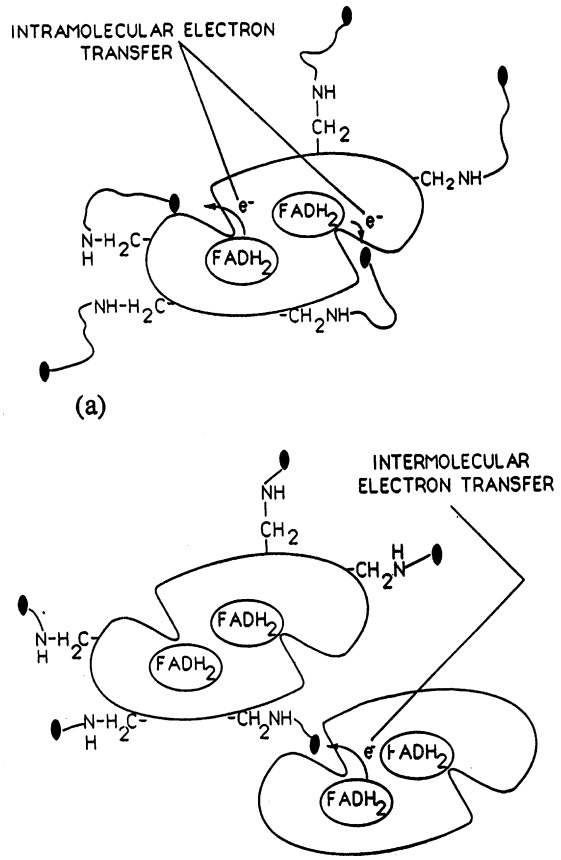


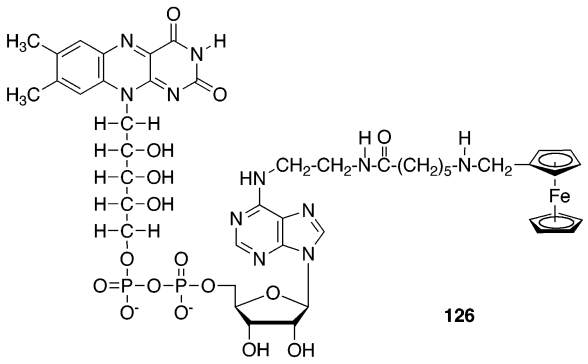
Figure 9. Two different mechanisms of electron transfer between ferrocene and $FADH_2$ in ferrocene-modified GOD (intramolecular and intermolecular). Reprinted with permission from ref 269. Copyright 1991 The American Chemical Society.

chains are consistent with those obtained by derivatization with various (short-chain) carboxylic acid derivatives presented above.

A few years later, Karube et al. also introduced aldehyde groups on the sugar residues of GOD and, subsequently, connected ferrocenyl moieties on adipic acid hydrazide spacers to the enzyme.[278−280] Also in these cases, direct electrical communication between the enzyme and the electrode took place. In addition, a mixture of the modified GOD derivative with organic solvent was used as the ink component for the screen-printing technique.

Katz, Willner, and co-workers divised a different strategy to decrease the distance between the FAD cofactor and the redox active ferrocene moiety. Instead of attaching ferrocene moieties randomly on the periphery of the enzyme, they reconstituted GOD from apo-GOD in the presence of a semisynthetic FAD cofactor **126** to which a tail bearing a ferrocenyl moiety is tethered.[281,282] The modified cofactor is depicted in Scheme 42. By performing electrochemi-

Scheme 42. Molecular Structure of the Ferrocenyl-Modified FAD Cofactor 126



cal and kinetic analyses, the rate for electron transfer between the modified enzyme and the electrode was found to be 40 s^{-1}.[282] This value is about 45 times higher than the highest observed rate constant for GOD randomly substituted on the periphery with ferrocene moieties ($k = 0.9$ s^{-1}). In conclusion, the enzyme reconstituted with the ferrocenyl-modified FAD cofactor exhibits superior electrical communication with respect to the randomly derivatized conjugates.

Chen et al. modified GOD on the gene level in order to attach a Lys_{10} tail via a $(Ser\text{-}Gly)_5$ peptide linker to the C-terminus and expressed the recombinant protein in the yeast *Pichia pastroris*.[283] Because translation proceeds from the N-terminus to the C-terminus, it was anticipated that the Lys_{10} tail would not be buried in the core of enzyme. In addition, the X-ray crystal structure of native GOD showed the C-terminus to be located at the surface of the wild-type (wt) protein, whereas the N-terminus is buried deeply in the core of the protein.[261] Moreover, the added Lys residues are hydrophilic and should prefer a position on the surface over a position in the hydrophobic core of the protein. Indeed, these lysine-$N_\epsilon H_2$ moieties were found to be accessible for labeling. After derivatization with **40** and EDC in 2 M urea, the modified Lys_{10}-GOD derivative displayed 90% activity relative to wt GOD. Furthermore, the Lys_{10}-GOD-Fc conjugate was found to have a better electrical availability and a higher lifetime than the ferrocenyl-conjugated GOD controls lacking the Lys_{10} tail.[283]

The group of Katz and Willner adapted modified GOD to probe antigen−antibody associations at the interfaces of the monolayer and the electrode and developed amperometric immunosensor electrodes.[69,273,275,284−287] The field of bioelectronics has been reviewed by this group.[70,288]

Aizawa et al. prepared GOD derivatives that were conjugated with both ferrocenyl moieties and digoxin.[289] The authors used two different procedures for ferrocenyl labeling: (1) the Fc-COOH/EDC protocol and (2) labeling via imine formation with Fc-CHO and subsequent $NaBH_4$ reduction. Conjugates with various Fc/digoxin/GOD ratios were prepared, and these were adapted in a homogeneous electroenzymatic immunoassay.

3.1.2. D-Amino Acid Oxidase (DAAO)

D-Amino acid oxidase (DAAO) was one of the first flavoproteins to be discovered in the 1930s.[290,291] The enzyme is found in numerous organisms, such as yeast, insects, birds, and mammals.[292] It catalyzes the oxidative deamination of D-amino acids according to the following equations (eqs 4−6) with the help of a FAD cofactor.

$$\mathrm{DAAO(FAD) + H_2N{-}CHR{-}COOH \rightarrow DAAO(FADH_2) + HN{=}CR{-}COOH} \quad (4)$$

$$\mathrm{HN{=}CHR{-}COOH + H_2O \rightarrow R{-}C(O){-}COOH} \quad (5)$$

$$\mathrm{DAAO(FADH_2) + O_2 \rightarrow DAAO(FAD) + H_2O_2} \quad (6)$$

In the first step, the amino acid is reduced to an imine (eq 4), which subsequently hydrolyzes to yield a keto ester (eq 5). Similar to the regeneration of glucose oxidase, $FADH_2$ is regenerated by dioxygen to return the DAAO in the FAD state (eq 6).

In vitro, DAAO is reactive toward a wide range of neutral and basic D-amino acids, but the highest reactivity is displayed toward amino acids that possess a hydrophobic side chain. The amino acids D-aspartic acid and D-glutamic acid are not substrates for DAAO, but these are instead oxidatively deaminated by D-aspartate oxidase. An excellent review on all aspects of DAAO recently appeared in the literature.[293]

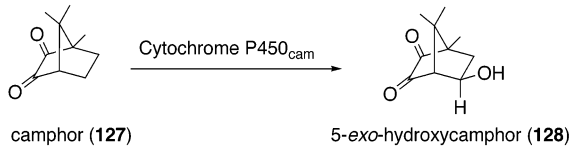
D-Amino acid oxidase from pig kidney (pkDAAO) was the first DAAO to be obtained in pure form in 1973.[294] About 15−20 years later, the isolation and purification of DAAO from the yeasts *Rhodoturula gracilis* (rgDAAO) and *Trigonopsis variabilis* (tvDAAO) were reported.[295,296] The enzyme pkDAAO is a monomeric 347 amino acid protein, containing one FAD cofactor, with a molecular mass of 39.6 kDa. The rgDAAO is a homodimeric enzyme of approximately 80 kDa, with each monomer consisting of 368 amino acids. Furthermore, the rgDAAO contains a noncovalently bound FAD cofactor.[297] Protein X-ray crystal structures of pkDAAO[298−302] and rgDAAO[303,304] have been reported.

Heller and Degani could bind 3 ± 1 ferrocenoyl moieties on pkDAAO by reaction with **40** in the presence of the coupling reagent EDC and 3 M urea.[267,268] The resulting conjugate showed about 25% activity relative to the native enzyme. Katz, Willner, and co-workers reconstituted apo-pkDAAO in the presence of the ferrocenyl-modified cofactor **126**. The reconstituted enzyme was reported to display only about 20% of activity relative to the native pkDAAO.[281] For both conjugates, it was demonstrated that direct electrochemical communication took place between the electrode and the modified enzyme. The addition of D-alanine to either conjugate resulted in an electrochemical response, with the magnitude of the current depending on the concentration of D-alanine. In fact, a linear relationship was observed between the D-alanine concentration and the current, thus making the use of the modified enzymes in amperometric D-amino acid sensors possible.[267,268,281]

3.1.3. Cytochrome $P450_{cam}$

Cytochrome $P450_{cam}$ ($CyP450_{cam}$) from the soil bacterium *Pseudomonas putida* is a monooxygenase with a molecular mass of 45 kD, which catalyzes the stereospecific hydroxylation of camphor **127** to 5-*exo*-hydroxycamphor (**128**) (Scheme 43).[305] For this transformation, it relies on two redox partners that transfer electrons from NADH to $CyP450_{cam}$, namely putidaredoxin, a redoxin with an $Fe_2S_2Cys_4$ structural motif, and putidaredoxin reductase. Like all members of the CyP450 family, $CyP450_{cam}$ contains a heme subunit, with the iron atom being coordinated by a planar tetradentate heme ligand and by a cysteinato-S atom. A variety of crystal structures of $CyP450_{cam}$ have been reported by Poulos and coworkers, including the substrate-free enzyme[306,307] and the enzyme complexed with its natural substrate camphor[308] and with several other substrate analogues.[309,310] In addition, crystal structures of $CyP450_{cam}$ inhibited by carbon monoxide[311] as well as by several other inhibitors have been reported.[312,313] The electrochemical and spectroscopic properties of $CyP450_{cam}$ have been extensively studied, and they were correlated with the results from protein X-ray crystallography.[314]

Scheme 43. Stereospecific Hydroxylation of Camphor 127 by Cytotyrone $P450_{cam}$



camphor (**127**) 5-*exo*-hydroxycamphor (**128**)

In a series of papers, Hill, Di Gleria, Wong, and co-workers prepared cytochrome $P450_{cam}$ derivatives modified with ferrocenyl maleimide residues.[212,315,316] The maleimide moiety has a high affinity and selectivity for the sulfhydryl group of cysteine (see section 2.4.4). The highlight of these papers is undoubtedly the protein X-ray single-crystal structure at 2.2 Å resolution of the $CyP450_{cam}$(C334A) mutant to which two ferrocenylethyl maleimide (**104**) groups were covalently attached.[315] The exposed Cys334, which is at the surface of the wt protein, was altered to an alanine via site-directed mutagenesis to prevent dimerization of the enzyme via disulfide formation. The overall structure of the modified enzyme is depicted in Figure 10.

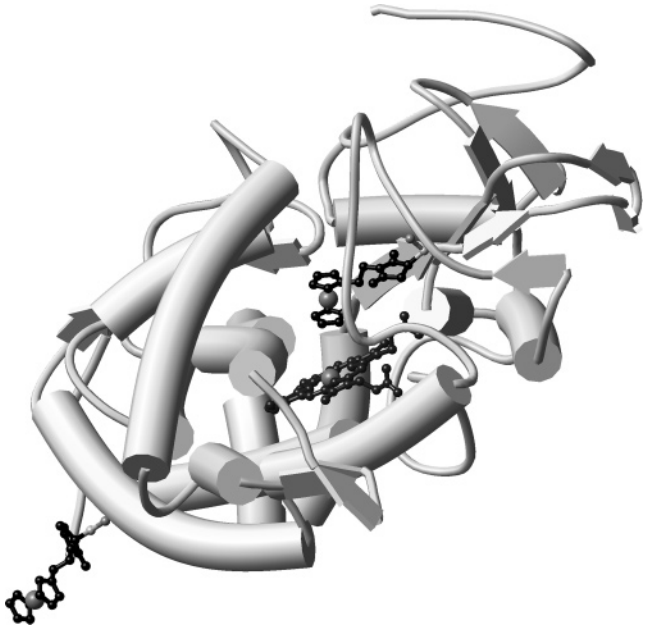


Figure 10. Protein X-ray single-crystal structure at 2.2 Å resolution of the $CyP450_{cam}$(C334A) mutant with two ferrocene groups covalently attached. One ferrocene group is located close to the hemin group in the center of the enzyme, while the other ferrocene is on the outside of the enzyme (bottom left). Graphics created from PDB entry 1GJM using WebLab Viewer Lite Vers. 3.2 on a Macintosh G4 Powerbook Computer.

This X-ray crystal structure analysis revealed that one of the ferrocenylethyl maleimide groups is tethered to Cys136 on the periphery of the enzyme, whereas the other maleimide moiety is covalently linked to Cys85, occupying a position in the binding pocket of the enzyme. The Cys85-bound ferrocenyl moiety turned out to be an irreversible inhibitor of the enzyme. By UV spectroscopy, it was shown that camphor is displaced from the binding pocket during the derivatization reaction of the enzyme camphor complex with **104**. UV spectroscopic results also indicated the simultaneous binding of a sixth ligand, most likely a water molecule, to the heme iron atom during the derivatization procedure. This is consistent with the results from X-ray crystallography, which show the binding of an aqua ligand to the heme iron atom. The binding of camphor is inhibited as a result of the occupation of the active site by the relatively bulky Cys85-bound ferrocenyl group. The heme iron atom, however, is still accessible for small molecules such as carbon monoxide. This could by verified by the appearance of a band at 448 nm in the UV spectrum upon reduction of the enzyme and exposure to CO. This Soret band is characteristic for the Fe(II) carbonmonoxy form of the enzyme.[314]

Another mutant of $CyP450_{cam}$ was prepared via site-directed mutagenesis.[316] All of the surface cysteines (Cys58, Cys85, Cys136, and Cys334) were changed to alanines, and in addition, lysine 344 on the periphery of the enzyme was changed into a cysteine. Subsequently, this K344C mutant was modified with *N*-ferrocenyl maleimide (note that this is a different maleimide derivative from the one used for the protein modification mentioned above). In contrast to the doubly labeled enzyme presented above, this conjugate was found to exhibit about 80% activity compared to wt $CyP450_{cam}$, as determined by NADH turnover rates.[316]

At this stage it should be noted that attempts have been made to modify bakers' yeast CyP450 with chloromercuriferrocene (**108**).[317−319] Instead of attachment of the ferrocenylmercury group to Cys102, a protein monomer modified at Cys102 with a $HgCl^+$ and a protein dimer, in which a Hg atom bridges the thiolate groups of two Cys102 residues, formed.

3.1.4. Horseradish Peroxidase

Horseradish peroxidase (HRP) is a monomeric heme-containig glycoprotein, consisting of 308 amino acid residues.[320] The activity of the enzyme relies on the presence of Ca^{2+} ions, which regulate the structure of the protein.[321,322] The X-ray crystal structure of recombinant HRP determined at 2.15 Å resolution was recently reported.[323] The enzyme reacts with alkylperoxides in the following manner, according to a modified ping-pong mechanism (eqs 7−9).

$$\text{HRP} + \text{R-O-O-H} \rightarrow \text{HRP-O(state } 2^+) + \text{ROH} \quad (7)$$

$$\text{HRP-O(state } 2^+) + \text{D}_{red} \rightarrow \text{HRP-O(state } 1^+) + \text{D}_{ox} \quad (8)$$

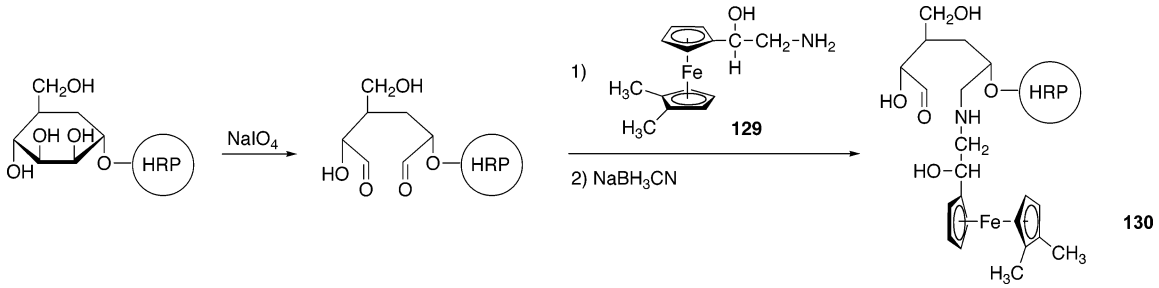
$$\text{HRP-O(state } 1^+) + \text{D}_{red} \rightarrow \text{HRP} + \text{D}_{ox} + \text{H}_2\text{O} \quad (9)$$

For HRP, only a narrow range of molecules can act as an oxidant, whereas a large variety of enzymes or molecules can act as the reductant (D_{red}). Equations 8 and 9 are one-electron reductions for HRP-O, and several ferrocene derivatives have been shown to be very suitable electron-donor substrates D_{red}.[324−328]

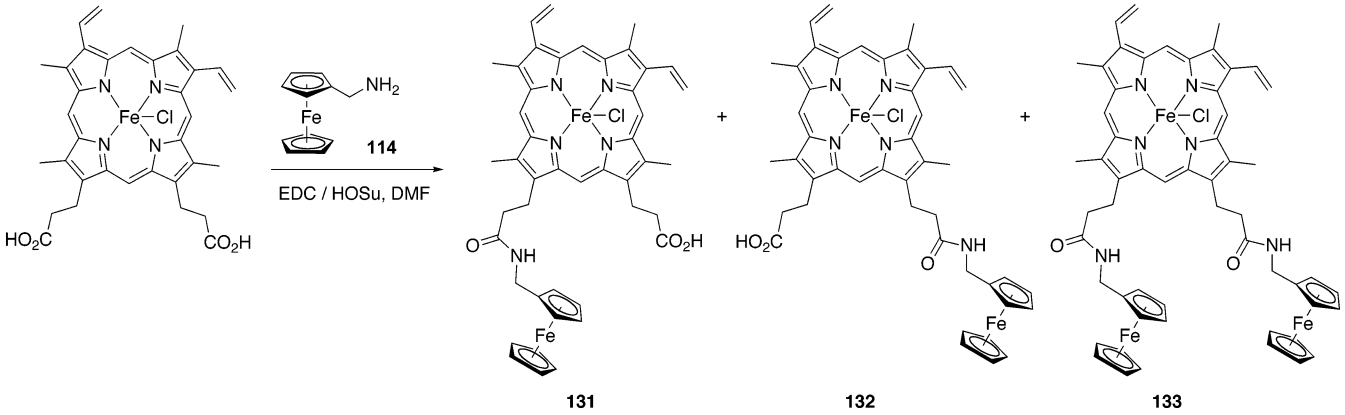
Tsai and Cass prepared HRP derivatives with a ferrocenyl moiety covalently attached to oxidized mannose residues of the main carbohydrate chain.[329] The strategy for the modification is outlined in Scheme 44. First, aldehyde groups are generated by $NaIO_4$ oxidation of the terminal mannose moieties. Next, imines are formed between the aldehyde and the ferrocenylamine (**129**), followed by reduction with $NaBH_3CN$ to yield the corresponding secondary amine HRP conjugate **130**. The kinetic parameters for this HRP−ferrocene conjugate are, in the case of H_2O_2 as the substrate, in the same range as those for unmodified HRP. With linoleic hydroperoxide as the substrate, however, differences between ferrocene-modified HRP and wt HRP were observed. The modified enzyme shows a significantly lower apparent K_m value and a significantly higher apparent V_{max} compared to the native enzyme. An explanation for the difference could be that the surface of the modified enzyme is more lypophilic than that of the unmodified enzyme. An enzyme electrode was constructed by absorbing the ferrocene HRP conjugate on a printed carbon electrode. This enzyme electrode was suitable for determination of H_2O_2 in the range 1−50 μmol/L and quantification of linoleic hydroperoxide in the range 5−100 μmol/L.

Ryabov and co-workers used a different method to introduce an electrochemically active ferrocene moiety to HRP.[330] This strategy consists of derivatization of hemin chloride with ferrocenylmethylamine (**114**), by using EDC/HOSu as the coupling reagent mixture

Scheme 44. Modification of the Mannose Side Chain



Scheme 45. Synthesis of Ferrocenyl−Hemin Chloride Conjugates 131−133



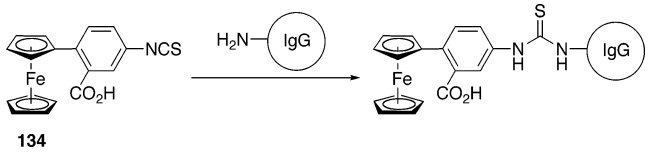
(see Scheme 45). In addition to two different isomeric monosubstituted derivatives **131** and **132**, also the disubstituted conjugate **133** was obtained. The mono- and disubstituted compounds could be separated by column chromatography, but separation of the two different monosubstituted positional isomers **131** and **132** was not possible. Reconstitution of apo-HRP in the presence of either the mixture of **131** and **132** or **133** alone yielded a catalytically active enzyme only with the mixture of the monosubstituted (**131/132**) hemin cloride derivatives. Kinetic measurements on ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) as a substrate revealed that **131/132** reconstituted HRP displayed the same Michaelis constant as native HRP. This indicates that substrate binding is not influenced by the presence of mono-ferrocenyl-modified heme. The V_m value for HRP reconstituted with **131/132** is about one-third of the V_m value for native HRP, which means that the catalytic activity of the modified enzyme is about 33% of that of the native HRP. When water-soluble ferrocene derivatives serve as electron donors, two differences between HRP containing **131/132** and native HRP are observed: (1) a change in the rate law and (2) an apparent increase in catalytic activity for HRP reconstituted with **131/132**. Results from molecular modeling, by starting from the X-ray crystal structure of recombinant HRP,[323] showed that the ferrocenyl rest is likely situated on the surface of the active site. It is believed that a more hydrophobic binding pocket is created on the modified enzyme, contributed by the ferrocenyl rest and the phenyl rings of two phenylalanine residues. This might explain the altered kinetics of the modified enzyme compared to native HRP in the case of ferrocene compounds serving as electron donors.

3.2. Nonredox Proteins

The first reports of synthetic polypeptides and proteins being labeled with the ferrocene moiety date back to the years 1966 and 1967.[331−334] As labeling reagents, ferrocenylmethyl isothiocyanate,[331−333] ferrocenylpropionic acid with carbodiimide as the activating reagent,[332] and ferrocenesulfonyl chloride (Fc-SO_2-Cl)[334] were used.

In 1967, a new type of reagent, namely *p*-ferrocenylphenyl isothiocyanate and its carboxylic acid derivative 3-carboxy-4-ferrocenylphenyl isothiocyanate (CFPI, **134**, Scheme 46), was introduced for

Scheme 46. Labeling of Immunoglobulin G with CFPI (134)

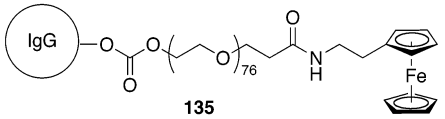


conjugation with primary amino groups.[335−337] These compounds were applied for histochemical purposes; that is, the covalently bound ferrocene moiety was visualized and located via fluorescence microscopy or, more efficiently, via electron microscopical methods. The usefulness of this technique was first demonstrated by labeling various tissues, such as the placenta,[338−341] and several proteins.[340−342] Thereafter, in particular the more polar carboxy derivative CFPI found application for the labeling of immunoglobulins, which will be treated in the next section.

3.2.1. Immunoglobulins

The labeling of antibodies, in particular IgG immunoglobulins, with CFPI has been reported in a

Scheme 47. Structure of the Poly(ethylene glycol) Linkers Attached to IgG Immunoglobulins



135

series of papers by Franz, Wildführ, Wagner, and coworkers in the late 1960s and early 1970s (Scheme 46).[342−348] The CFPI-labeled immunoglobulins were used for immunohistochemical investigations or, in other words, the electron microscopical visualization of hapten (or antigen) sites. In a series of papers, the concept of this immunohistochemical method, in combination with a detailed description on how labeling of immunoglobulins could be achieved, was presented.[347,348,344] In subsequent papers, CFPI-labeled immunoglobulins were adapted for the localization and visualization of (1) cell wall antigens, in this case the M protein, in the bacterium *Streptococcus pyogenes*,[343] (2) surface antigens on the parasite *Toxoplasma gondii*,[346] and (3) GRAFFI-virus-induced surface antigens in rat GRAFFI leukemia cells. In these three cases, the CFPI immunoglobulins could be satisfactorily visualized when they were bound to the antigen.[345]

In addition to derivatization with CFPI, examples of immunoglobulin labeling with the reagents chloromercuriferrocene (**108**),[319,349] ferrocenecarboxylic acid (**40**),[350−356] ferrocenylacetic acid (**124**),[357] and ferrocenecarbaldehyde (**67**)[358] have also been reported. Yasuda attached **67** to amino groups of the immunoglobulin via imine formation and subsequent $NaBH_4$ reduction to yield antibodies labeled with the Fem group.[358] However, these labeled immunoglobulins were not well characterized and their properties were not determined.

In another paper by Yasuda and Yamamoto, ferrocene labels were introduced in an indirect manner on the immunoglobulin, by using ovalbumin as a ferrocene-label-carrying molecule (see also section 3.2.3).[349] First, hen's egg ovalbumin was reacted with *N*-acetylhomocystein thiolactone, which resulted in amide formation between protein NH_2 groups and the C-terminus of the *N*-acetylcystein. In this way, about nine new sulfhydryl groups were introduced on the protein ovalbumin. Subsequently, ferrocene moieties were covalently attached through Fc-Hg-S bond formation of chloromercuriferrocene (Fc-HgCl, **108**) with sulfhydryl groups. Next, aldehyde groups were introduced on the carbohydrate part of the ovalbumin derivative via reaction with $NaIO_4$. In the last step, the ovalbumin−HgFc conjugate was covalently attached to immunoglobulins via imine formation, followed by reduction with $NaBH_4$.

Only in the past decade, ferrocenecarboxylic acid (**40**) and ferrocenylacetic acid (**124**) have been used to label various immunoglobulins after activation of the carboxylic acid with EDC or related reagents.[350−357] These conjugates were used for immunoassays with electrochemical detection either following capillary electrophoresis or immobilized on gold surfaces. For instance, histamine concentrations between 200 and 2000 ng/mL could be reliably determined within 2 min from whole blood samples using a microfabricated on-chip multichannel matrix column device.[354]

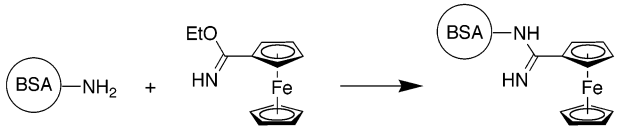
In a series of interesting papers, Moiroux and coworkers labeled IgG immunoglobulins with PEG-Fc molecules to yield conjugate **135** (Scheme 47).[359−361] The ferrocene-containing chain of **135** with average weight $M = 3400$ is very flexible. They assembled layers of modified IgG molecules and anti-IgG antibodies on electrodes and determined several physical properties, such as the diffusion of the redox probes and the dynamics of the chain.

Signal amplification was achieved in an amperometric immunosensors by Yasuzawa and co-workers. The anti-transferrin antibody 15C2H3 was labeled with a conducting ferrocene-containing polymer **136** (HO-poly-Fc, Scheme 48). This polymer is coupled to amino groups of the immobilized antibody, and a quantitative analysis of transferrin is carried out by measuring the electrooxidation current of ferrocene, which is proportional to the transferrin concentration.

3.2.2. Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is a protein with a molecular weight of 66.7 kD, consisting of 607 amino acids, of which 57 are lysine residues.[362] The first report of it being labeled with a ferrocene derivative appeared in the literature in 1969.[363] Schlögl et al. reacted BSA with ferrocene imidocarboxylic acid ethyl ester (**137**) (Scheme 49), resulting in a conjugate

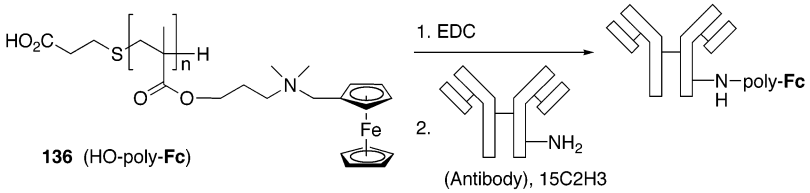
Scheme 49. Labeling of BSA with Ferrocenylimidocarboxylic Acid Ethyl Ester (137)



137

that, according to the authors, contained approximately 60 ferrocenyl groups. This value should be taken with appropriate care, because it has been determined by gravimetry and not by more accurate techniques such as atomic absorption spectrometry (AAS) or spectrophotometry.[364] As a matter of fact, BSA contains only 57 lysine amino groups.[362]

Scheme 48. Synthesis of a Ferrocene Polymer Anti-transferrin Antibody



136 (HO-poly-**Fc**)

(Antibody), 15C2H3

In two papers, Mizutani and Asai introduced Fem groups on the N_ϵ atom of the lysines, via the reaction with ferrocenecarbaldehyde (**67**) followed by $NaBH_4$ reduction.[365,366] Under denaturing conditions in the presence of 6 M urea, about 40 of the 57 lysine N_ϵ atoms were derivatized, whereas, without added urea, only 20 Fem rests were introduced.[366] These ferrocenyl−BSA conjugates were applied as efficient diffusing macromolecular mediators between the enzyme glucose oxidase and the electrode.

Shinohara and co-workers reacted BSA with ferrocenylpropionic acid in the presence of EDC, which resulted in formation of two types of conjugates: proteins that possessed either 23 or 5 ferrocenyl rests.[367] The authors showed these conjugates to be efficient macromolecular mediators between the enzyme fructose dehydrogenase and an electrode. The conjugate with higher ferrocene/BSA ratio was found to be more effective, which was explained by the higher numer of redox centers attached.

In an interesting paper, Kunugi et al. explored the reaction of BSA with **67** followed by $NaBH_4$ reduction under different conditions. They investigated the differences between the denaturation of the protein via high pressure (500 MPa) or via urea concentrations ranging from 3 to 6 M during the labeling process. Conjugates with higher ferrocene/BSA ratio were obtained by applying a 500 MPa pressure. However, the conjugates prepared via denaturation with urea were better mediators between GOD and the electrode, despite the lower number of ferrocenyl rests attached.[274]

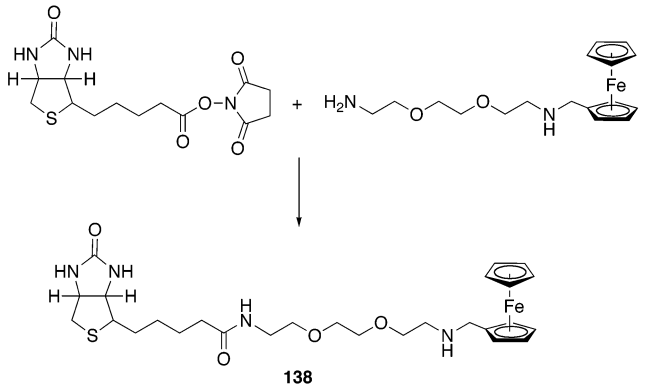
Aizawa and co-workers prepared ferrocene and digoxin double-conjugated BSA derivatives for application in a homogeneous electrochemical immunoassay.[368] In total, 39 Fem groups and 8 digoxin molecules were connected to BSA via imine formation and subsequent $NaBH_4$ reduction. This derivative was used as a multisite hapten for digoxin antibodies. Binding of the digoxin antibodies results in a decrease of the electrochemical activity of the BSA derivative. This is caused by the diminished electrochemical availability of the ferrocene rests, since the bulky antibodies shield the ferrocene moieties. The measured peak height currents are inversely related to the concentration of antibodies, in this way allowing the quantification of the antibodies.

Lo and co-workers have also used ferrocenyl iodoacetamide (**105**) for the labeling of sulfhydryl-modified BSA.[214] Before reaction with **105**, BSA was treated with *N*-succinimidyl-*S*-acetylthioacetate to increase the number of free thiol groups. After the labeling, the ferrocene-to-BSA ratio was about 40:1, as estimated from UV absorbance spectra.

3.2.3. Various Proteins

The first report of a protein, in this case ovalbumin, labeled with the ferrocene rest dates back to 1967.[334] Ovalbumin, the main protein of egg white, is a glycoprotein with a molecular mass of 45 kD.[369] The primary amino acid sequence of hen ovalbumin has 385 residues, of which 20 are lysines,[370,371] and the N-terminus of the protein is acetylated.[372] The crystal structure of uncleaved ovalbumin was determined at 1.95 Å resolution.[373] In a short communication, Peterlik reported the reaction of ferrocenesulfonyl chloride with ovalbumin to yield conjugates in which on average 8.6 of the total 20 lysines were derivatized.[334]

Scheme 50. Ferrocenyl Biotin Conjugate 138



138

The protein papain found in unripe fruit (papayas) of the tree *Carica papaya* belongs to the class of cysteine proteinases.[374] This protein consists of a single chain of 212 amino acids, of which 7 are cysteines.[375−377] The X-ray crystal structure of the protein revealed that 6 of these cysteines form disulfides, whereas Cys25 is located in the active site.[378] This Cys25 is essential for enzyme activity.[374] Douglas and co-workers performed experiments to investigate whether chloroacteylferrocene (**41**) or chloromercuriferrocene (**108**) could irreversibly inactivate papain.[379] The latter reagent turned out to be completely unsuitable, whereas **41** rapidly inactivates papain. Kinetic experiments indicated that the inhibition occurs via a two-stage process, involving initial complexation of **41** by the enzyme, followed by covalent attachment via thioether formation.

The tetrameric proteins avidin (from egg white) and streptavidin (from the bacterium *Streptomyces avidinii*) are remarkable for their ability to bind up to four molecules of biotin with high affinity (K_d = 10^{-15} M for avidin and $K_d = 10^{-13}$ M for streptavidin).[380,381] Although these proteins are genetically remote, their primary structures are rather similar[382] and the conserved amino acid residues are mostly confined to six homologous segments.[383,384] Protein crystal structures have been reported of avidin[385,386] and streptavidin[387,388] with and without bound biotin.

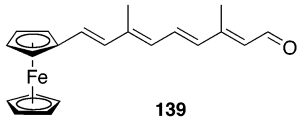
Padeste and co-workers coupled the ferrocene moiety on a flexible spacer via an amide linkage to avidin as well as to streptavidin, by applying EDC as the coupling reagent.[389,390] By controlling the reaction conditions, avidin and streptavidin conjugates with Fc/protein ratios varying from 3 to 30 (for avidin) and from 3 to 16 (for streptavidin) were obtained.[389,390] The derivatization procedure did not alter the biotin-binding capabilities of the enzyme significantly. These ferrocenyl−avidin conjugates were used to construct an enzyme-based biotin sensor by immobilizing them on an electrode.[389]

In another paper, Mosbach and Schuhmann followed a different approach toward an affinity-based biotin sensor, by making use of the biotin−streptavidin system. Instead of derivatizing the enzyme, they prepared a ferrocene−biotin conjugate **138**

(Scheme 50).[391] The affinity assay was based on the fact that the measured current for a microelectrode is correlated to the diffusion coefficient of the redox species. Binding of the electroactive conjugate **138** to streptavidin results in a change of the diffusion coefficient of the electroactive label on account of the increase in molecular mass. Suggestions for signal amplification were given in this paper, and the generality of the idea was demonstrated.

Khodonov and co-workers prepared a ferrocenyl analogue of retinal **139** and tested its interaction with bacteriorhodopsin (BR) from *Halobacterium salinarum* (Scheme 51).[392] Bacteriorhodopsin, discovered

Scheme 51. Ferrocenyl Retinal Conjugate 139



139

in 1971 by Oesterhelt and Stoekenius,[393] is a membrane protein. Its protein component, called bacterioopsin, consitutes a single polypeptide chain of 247 amino acids, with a lysine in position 216. The chromophore retinal is attached via an imine linkage to Lys216, with the aldimine fragment being protonated.[394−396] Compound **139** was reacted with bacterioopsin apomembranes, and a stable pigment formed slowly. UV−vis investigations showed that the λ_{max} of the **139** reconstituted bacterioopsin is nearly the same as that for the native BR (492 vs 483 nm, respectively).

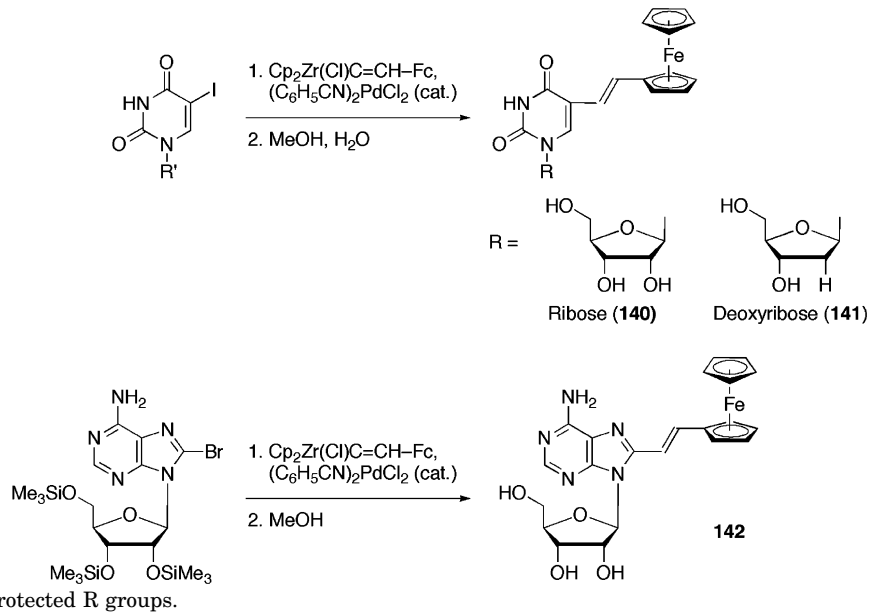
4. Conjugates of Ferrocene with DNA, RNA, and PNA

Compared to the ferrocene derivatives of amino acids and proteins described in section 2, this is a relatively young and less explored field in the bioorganometallic chemistry of ferrocene. It is interesting to note, however, that most of the chemistry and applications of ferrocene are rather similar. Imine and amide formation with ferrocenecarbaldehyde (**67**) or ferrocenecarboxylic acid (**40**), respectively, as well as Sonogashira coupling are the preferred methods for covalent attachment of ferrocene to (oligo)nucleotides. A potentially very promising application is simple and inexpensive gene sensors.[397] For such devices, electrochemically active DNA derivatives for sequence-specific detection of DNA oligomers are required. Unsurprisingly, most efforts are concentrating on ferrocene derivatives for this purpose. As will be discussed later (section 4.3), a variety of detection schemes are conceivable and there does not seem to be a gold standard yet. Grinstaff and coworkers have summarized metallo-oligodeoxynucleotides[398] and recent developments in redox probes for DNA.[399] The subject of this section has recently been reviewed.[71]

4.1. Ferrocene Derivatives of Nucleobases, Nucleosides, and Nucleotides

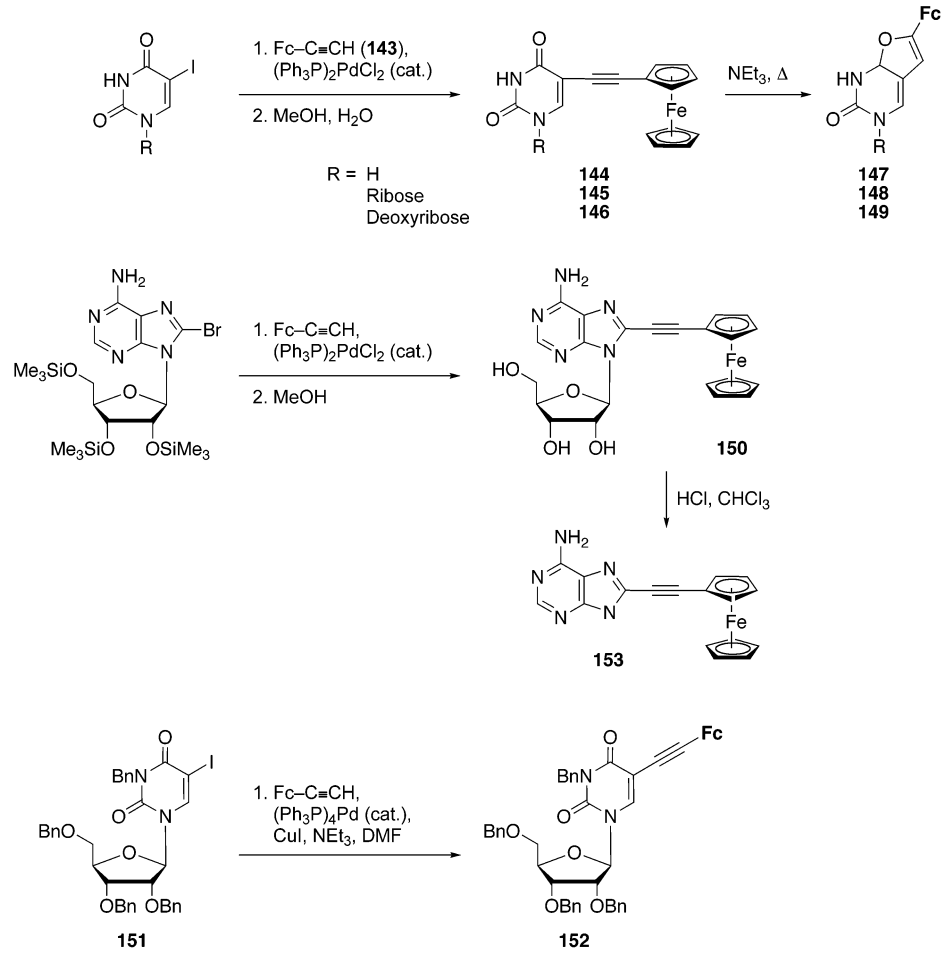
The first work on ferrocene derivatives of nucleosides is only about 10 years old.[400] Moreover, this work by a French group has not been cited frequently again, but obviously, some "first" or "unprecedented" claims in later papers are unjustified. Gautheron and co-workers used a variety of different Pd-catalyzed C−C coupling reactions to synthesize ferrocene nucleosides and derivatives thereof.[400] The reaction of unprotected 5-chloromercury-uridine with ethenylferrocene yields, under Pd catalysis and after reductive workup, a mixture of ferrocenyluridine derivatives. A cleaner reaction was observed with TMS-protected 5-iodouridine, 8-bromoadenosine, and 2′-desoxyuridine using the zirconylated compound $Cp_2Zr(Cl)CH{=}CH{-}Fc$ (derived from ethynylferrocene and Schwartz's reagent $Cp_2Zr(Cl)H$) and $(C_6H_5CN)_2PdCl_2$ as a catalyst. After workup with aqueous MeOH, the unprotected uridine, 2′-desoxyuridine, and adenosine derivatives **140**−**142** were obtained in good yield (Scheme 52). A reaction is also possible with

Scheme 52. Pd-Catalyzed Synthesis of Ferrocenylated Nucleosides[a]



[a] R′ denotes Me_3Si-protected R groups.

Scheme 53. Sonogashira Coupling for Ferrocenylated Nucleosides

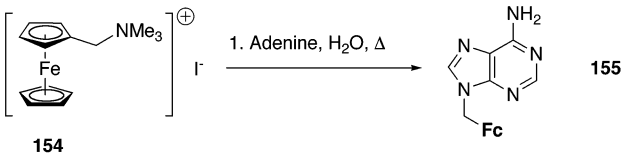


ethynylferrocene (**143**) itself and 5-iodouracil, iodouridine, 2-desoxyiodouridine, and bromoadenosine (Sonogashira coupling),[186] yielding the substituted nucleobase 5-ethynylferrocenyluracil (**144**), 5-ethynylferrocenyluridine (**145**), and 5-ethynylferrocenyldesoxyuridine (**146**) as well as the ethynylferrocenyladenosine (**150**) (Scheme 53). The 5-ethynyluracil derivatives can be cyclized in the presence of base, for example, NEt_3, yielding ferrocenyl derivatives **147**–**149**. These authors find that is not a metal-catalyzed reaction because the cyclization reaction takes place by simply heating ethynylferrocenyluracil in DMF in the presence of base.[400] The same observation was made later by Yu et al., and a mechanism has been suggested.[401] However, in a very recent publication, the presence of CuI was claimed to be essential in order for substantial amounts of **147** to be formed.[402] Two additional unexpected side products were found, all of which formed exclusively from **144**. An improved mechanism has been suggested which satisfactorily explains the range of products observed so far. In line with this, the protected nucleoside **151**, in which cyclization is not possible, yields the Sonogashira product **152** in 79% yield. Related cyclization reactions with purely organic substrates have previously been reported in the literature.[403–406] The ferrocenyl substituent stabilizes a positive charge on the directly bound ethynyl carbon atom,[407] thus facilitating an attack of the nucleophilic oxygen atom to form the five-membered ring. Houlton and coworkers also reinvestigated this cyclization reaction.[408] They reported crystal structures of protected derivatives of **146** and **149**. The formation of cyclization product **149** could be completely suppressed by running the Sonogashira reaction at room temperature for 4 h. Under these conditions, 5′-DMT-protected **146** could be isolated in 75% yield. Interestingly, a substantial amount of cyclization product was found after ODN synthesis on solid support and cleavage, even under very mild conditions using so-called ULTRAMILD phosphoramidites and 0.05 M methanolic K_2CO_3 for deprotection.[408] This result does indeed question the necessity of CuI in the cyclization reaction. Cleavage of the anomeric bond in the ferrocenyl adenosine derivative **150** could be affected by a solution of anhydrous HCl gas in chloroform to yield the ethynylferrocenyladenine (**153**).[400] In the course of this reaction, the typical blue color of ferrocenium cations was observed but the ethynylferrocenyladenine was recovered in good yield after workup. Finally, the cytotoxicity of selected compounds has been tested by a simple cell proliferation assay, but only modest effects were observed.[400]

Another approach to metal nucleobase derivatives was published by Houlton's group.[409,410] Trimethyl(ferrocenylmethyl)ammonium iodide (**154**) was used as a convenient source of the ferrocenylmethyl cation in the reaction with variuos nucleobases and nucleo-

base derivatives. A complete series of cytosine, thymine, uracil, guanine, and adenine derivatives was obtained and comprehensively characterized, also by X-ray analysis. In general, the presence of the lipophilic ferrocenylmethyl moiety makes those compounds less hydrophilic. Some of the compounds show interesting intermolecular hydrogen bonding patterns in the solid state, as revealed by X-ray single-crystal analyses. A representative example is shown in Scheme 54 for the case of 9-ferrocenylmethyladenine (**155**).[409] The first preparation of this compound was reported in 1980 by Chen, who actually obtained a mixture of **155** along with the isomeric N^6-ferrocenylmethyladenine and 7-ferrocenylmethyladenine.[411] The crystal structure of **155** reveals a combination of hydrogen bonding to Watson−Crick as well as Hoogsteen sites (Figure 11).

Scheme 54. Synthesis of 155



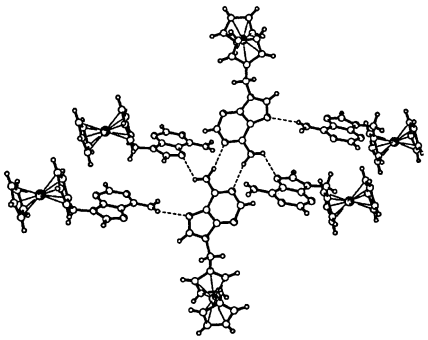
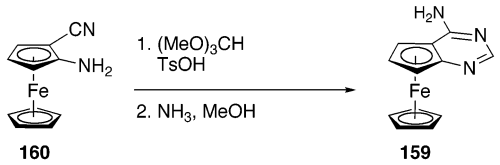


Figure 11. X-ray single-crystal structure analysis of **155**. Hydrogen bonds between the adenine rings are formed using Watson−Crick (two hydrogen bonds, central) and Hoogsteen sites (peripheral, one hydrogen bond each). Reprinted with permission from ref 409. Copyright 1996 The Royal Society of Chemistry.

An interesting application of hydrogen bonding involving nucleobases was reported recently. The distance between the Cp rings in ferrocene is about 3.5 Å. This corresponds almost exactly to the distance between stacked bases in dsDNA. Using this principle, a receptor **156** for the dinucleotide TpT was synthesized by Sonogashira coupling of 1,1′-diiodoferrocene (**157**) with 2 equiv of 2,6-diamido-4-ethynylpyridine (**158**) (Scheme 55).[412] An association constant of **156** (R = COMe) with a lipophilic TpT analogue of about 1.2×10^5 L/mol was calculated in $CHCl_3$. Moreover, an induced CD signal was observed resulting from absorptions of the ferrocene moiety. Because the receptor **156** is achiral, this induced CD signal can only be explained by a twisted orientation of the two Cp rings as a consequence of strong hydrogen bonding to the chiral TpT molecule. This situation resembles the work of Hirao, where hydrogen bonds between adjacent amino acids and peptides were forcing the substituted Cp rings on ferrocene into a fixed position, thus introducing an element of helical chirality.[154] Likewise, an induced CD was observed in both cases. The selectivity of the TpT receptor **156** over other mononucleotides or dinucleotides was also high. From a mixture of all conceivable mono- and dinucleotides, only TpT was extracted by the molecular receptor **156** (R = CO^nBu).[412] Although it is not clear how this principle can be extended to longer oligonucleotides, such selective receptors may certainly play a role in identification and purification of oligonucleotides in the future.

Compound **159**, which has planar chirality, represents a different approach to metallocene derivatives of nucleobases. **159** was synthesized regio- and stereospecifically by an eight-step procedure starting from ferrocenecarbaldehyde (**67**) protected with a chiral auxiliary.[413] The key intermediate 1-amino-2-cyanoferrocene (**160**) is cyclized with trimethylorthoformate and *p*-toluenesulfonic acid, followed by treatment with a solution of NH_3 in MeOH to yield **159** (Scheme 56). This compound represents an electrochemically active adenine analogue, in which the electroactive metallocene is actually part of the nucleobase itself. Whether such metallocene nucleobase derivatives might find any application remains to be seen.

Scheme 56. Synthesis of 159



4.2. Solid-Phase DNA Synthesis with Ferrocenylated Nucleotides

As pointed out before, the main interest in ferrocene oligonucleotide derivatives is for electrochemical DNA sensors. Therefore, most of the literature covers ferrocenyl oligomer synthesis under the aspect of electrochemical detection. However, a few papers

Scheme 55. Synthesis of a Molecular Receptor for the Dinucleotide TpT

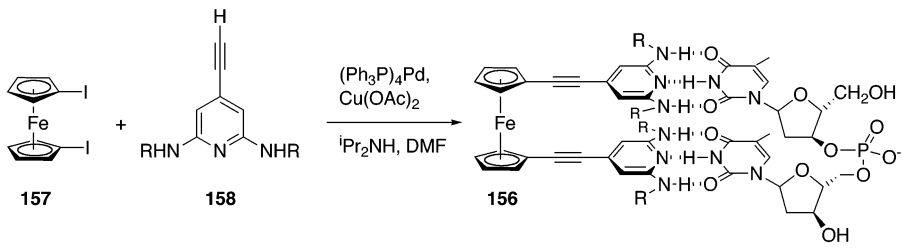


Table 6. List of Melting Temperatures of Ferrocenylated ODNs with Complementary DNA

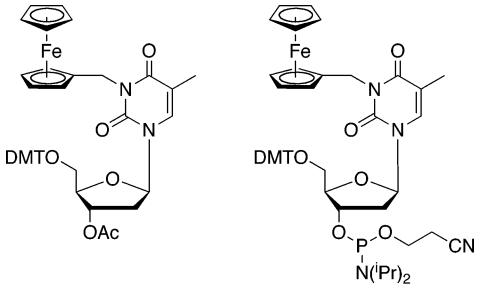
entry no.	length of oligomer	sequence	place of substitution with Fc	T_m[a]/°C	ΔT_m[b]/°C	ref
1	17	5′-CT^{Fc}G CTA GAG ATT TTT AC-3′	base	52.2 (compl DNA)	−0.4	414
				48.3 (compl RNA)	−7.7	
2	17	5′-CTG CTA GAG ATT^{Fc} TTT AC-3′	base	38.5 (compl DNA)	−14.1	414
				41.3 (compl RNA)	−14.7	
3	16	5′-TGC TA^{Fc}C AAA CTG TTG A-3′	base	43.2	−7.6	419
4	16	5′-TGC TAC A^{Fc}AA CTG TTG A-3′	base	41.8	−9.0	419
5	16	5′-TGC TAC AAA^{Fc} CTG TTG A-3′	base	42.4	−8.4	419
6	16	5′-TGC TAC AAA CTG TU^{Fc}G A-3′	base	49.6	−1.2	418, 419
7	16	5′-TGC TAC AAA CU^{Fc}G TTG A-3′	base	48.2	−2.6	418, 419
8	12	5′-TGC TAC AAA CU^{Fc}G-3′	base	42.6	+1.2	418
9	15	5′-ATC U^{Fc}GA GTC CAT GGT-3′	base	48.0 (A opposite U^{Fc})	−5.7	401
				56.5 (G opposite U^{Fc})	−4.2	
10	15	5′-AA^{Fc}C A^{Fc}GA GTC CAT GGT-3′	2′	51.3	+0.1	452
11	15	5′-AC^{Fc}A C^{Fc}TA GAG CCA GCT-3′	2′	57.4	−0.9	452
12	15	5′-ACA CTA GAG C^{Fc}CA GCT-3′	2′	58.7	+0.4	452
13	15	5′-AC^{Fc}A C^{Fc}TA GAG CCA GCT-3′	3′	53.7	−4.6	452
14	15	5′-ACA CTA GAG C^{Fc}CA GCT-3′	3′	54.2	−4.1	452
15	15	5′-AA^{Fc}C A^{Fc}GA GTC CAT GGT-3′	2′	51.3	+0.1	454
				50.5 (different Fc derivative)	−0.7	
16	37[c]	5′-CAA CGT CCG AGC AGT ACA T^{Fc}T^{Fc}G ACA GAC T^{Fc}AA GGA GCT^{Fc} T^{Fc}-3′	base	75	−4	423

[a] Values depend on oligomer concentration, added salts, salt concentrations, and melting schemes employed. In some cases but not all, multiple measurements were carried out and errors were given or estimated. Please refer to the original literature for those details. [b] ΔT_m is against a perfect match (T_m(ferrocenylated nucleotide) − T_m(unmodified nucleotide)). Only the site of labeling with ferrocene is indicated; different labels were used (see text and schemes). [c] The complementary strand was a 40-mer with an additional AGC triplet at the 3′ end.

deal with synthetic aspects alone, and these are covered in this section.

In one instance, Mitsunobu reaction of ferrocenylmethanol with 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-3′-*O*-acetylthymidine yields the coupling product **161**, which was readily converted to the phosphoramidite **162** (Scheme 57). Purified **162** was used as a building

Scheme 57. Ferrocenylated T Monomers 161 and 162[a]



161 **162**

[a] Compound **162** could be used as a modified T monomer in automated oligonucleotide synthesis.

block in automated oligonucleotide synthesis of DNA 16-mers and 17-mers.[414] Four different metalated oligomers were synthesized, differing in the position of the metal label. A series of melting studies with complementary DNA and RNA strands were performed. In all cases, a drastic reduction in the stability of the duplices was observed except when the metalated T was almost terminal in a B-DNA duplex (entry 1 in Table 6). Even the formation of triple helices was observed with the third strand bearing the metalated T. CD spectroscopic studies and CD melting point studies were further used to supplement structural information on the duplices and triplices formed in this study. This work represents the most comprehensive study to date on the influence of the site of ferrocene substitution on the stability and structure of DNA·RNA duplices, ds-DNA, and triplex formation.

Ferrocenecarboxylic acid (**40**) has been attached to the amine group of a 5′-terminal hexylamine of ODNs. In 1991, the purpose of these conjugates was to use stable isotopes of iron for multiplexed detection of nucleotides of different sequence by resonance ionization spectroscopy (RIS), either induced by ion sputtering (sputter-initiated RIS or SIRIS) or laser desorption (laser atomization RIS or LARIS).[415−417] This idea is somehow related to the very first metallo immuno assays on ferrocenyl derivatives of steroids reported by Cais, in which AAS detection was used (see section 6.1.1). Although the method worked with high spatial resolution on electrophoresis gels and the sensitivity was comparable to autoradiography with ^{32}P, its use is very limited. A relatively high background of ^{56}Fe was found in other carriers such as Nylon membranes, making the use of ^{57}Fe necessary for good sensitivity.[416] Second, a sophisticated and expensive device for detection is necessary. Finally, the advent of DNA chips has largely overcome the need for multiplexed analysis in traditional sequencing.

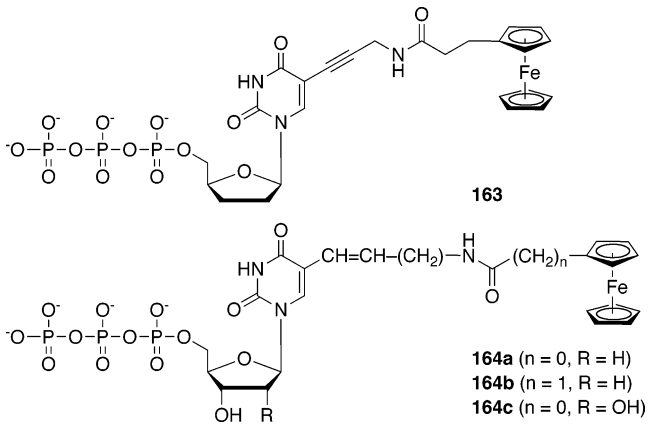
In most other work, ferrocenylated nucleotides were synthesized by Sonogashira coupling of ethynylferrocene (**143**) or related ferrocene derivatives with an ethynyl group to iodo-U or bromo-A. Two synthetic strategies have been followed, either transformation of ferrocenylated nucleotides into phosphoramidites and subjection to automated oligomer synthesis or derivatization with ethynylferrocene *after* assembly of the oligomer. Metzler-Nolte et al.

have found that ferrocenecarboxylic acid propargylamides **90** are particularly suitable ferrocene derivatives for Sonogashira coupling on amino acids and peptides (see Scheme 29 above). In addition, these compounds are conveniently prepared on a large scale, and the X-ray crystal structure of **90b** has been reported.[190] Compound **90a** was used by Grinstaff et al. for the labeling of nucleotides by Sonogashira coupling.[398,418,419] These authors reported improved yields and purity of the conjugates if the derivatization is carried out on the column, rather than in solution after cleavage of the oligonucleotide. Similar findings were made for labeling of DNA by Sonogashira coupling with a tris(bipyridine)Ru propargylamine derivative.[420,421] In their work, Grinstaff et al. found that the structure of dsDNA at room temperature is not significantly altered by incorporation of a ethynylferrocenyl-A or -U, regardless of the position of the label. On the other hand, a decrease in melting temperature of up to 9 °C (from 50.8 to 41.8 °C for a 16-mer) is observed if an A in the middle of one strand is substituted by ferrocene. Again, the effect is smaller if U is substituted and if the label is close to the end of the duplex.[419]

During their efforts to incorporate ethynylferrocene directly into U monomers by Sonogashira coupling, Yu and co-workers observed the same cyclization reaction in the presence of DIPEA that had already been reported by Gautheron and co-workers.[400,401] Both isomers **146** and **149** (see Scheme 53 above) could be separated, transformed into phosphoramidites, and used as building blocks in automated DNA oligomer synthesis. On the other hand, standard DNA deprotection/coupling requires basic conditions, and cleavage of (ferrocenylated) ODNs from the solid support is achieved by concentrated NH_3. It is, therefore, not surprising that only the cyclized ferrocenyl-U (**149**) is found in ODNs, regardless of the starting material, and this point is indeed convincingly demonstrated in this paper. However, another aspect of this work really is surprising. A number of hybridization experiments with 15-mer ODNs were carried out using ferrocenyl-U in the fourth position.[401] If (cyclized) Fc-U is opposite to A, T_m is 48.0 °C and the decrease in melting temperature ΔT_m is −5.7 °C compared to a perfect match. However, if G is placed opposite to Fc-U, the melting temperature is 56.5 °C, which is even higher than that of the perfect A−T match just mentioned. On the other hand, ΔT_m for a perfect G−C match is −4.2 °C, which seems a reasonable number. It can be concluded that cyclized Fc-U behaves more like dC and prefers to hybridize to G instead of A. This is an important finding because it underscores the necessity for comprehensive studies. A ferrocenyl nucleobase derivative—or indeed, any modified nucleobase—may alter the properties of that nucleobase to such an extent that it actually resembles a different base. As yet, in-depth investigations along these considerations are lacking altogether for ferrocenyl-modified ODNs.

In all cases reported so far, including the ones in the following section (section 4.3), the ferrocene label is introduced either on the monomer stage, by a metal-catalyzed reaction (Sonogashira), or after assembly of the oligomer sequence at the 5′ end. An interesting alternative for the labeling of the 3′ end of a oligonucleotide was presented by Anne and coworkers. These authors used the dideoxynucleotide triphosphate Fc-ddUTP (**163**) (Scheme 58) in an

Scheme 58. Ferrocenylated UTP Derivatives That Have Been Used in Enzymatic Oligonucleotide Modification Reactions



enzymatic reaction to extend the 3′ terminus of an oligonucleotide.[422] The building block **163** was prepared from 5-iodouridine and a ferrocenyl alkyne, followed by several standard organic synthesis steps in an overall yield of about 10%. The enzyme terminal deoxynucleotidyl transferase (TDT) was then used to extend 5′-$(dT)_{10}$ at the 3′ end. Rapid and quantitative transformation was observed by HPLC with a 10-fold excess of Fc-ddUTP. Wlassoff and King have tested two ferrocene dUTP derivatives **164a** and **164b** as substrates for common DNA polymerases (Scheme 58).[423] Interestingly, only **164a** was a good substrate, whereas the slightly longer **164b** is very poorly incorporated. These authors also convincingly demonstrated the higher sensitivity of electrochemical vesus UV detection for HPLC analysis of ferrocenylated ODNs.[423]

A general approach for introducing a label such as ferrocene has been published by Saito.[424] The aldehyde-containing universal base 3-formylindole 2′-deoxynucleoside is introduced in an ODN during solid-phase synthesis at any desired position. The ODN is then treated with a hydrazine or ferrocene carbohydrazide to yield the postsynthetically modified ferrocene ODN. Unfortunately, no additional data, for example, melting temperatures with complementary DNA or electrochemical characterization, were presented. On the other hand, this approach is quite universal and certainly deserves further attention.

4.2.1. Analysis of Duplex Stability with Ferrocenylated Oligomers

The paradigm of molecular biology states that the genetic information of any organism is stored in the DNA of that organism.[425] This DNA is a heteroduplex (double-stranded DNA, dsDNA) consisting of two nonidentical DNA single strands (ssDNA) which are complementary. Complementarity is achieved by pairing of opposite bases through hydrogen bonds. Perfect pairing is achieved in the so-called canonical

base pairs, that is, G−C and A−T (or A−U in the cases of a DNA·RNA duplex). In an ideal double helix, those pairs form three (G−C) or two (A−T) hydrogen bonds, a situation which is called Watson−Crick base pairing. Normally, even short (>15 bases) strands of dsDNA wind up in a helix. The thermal stability of such a helical dsDNA can be estimated by measuring its melting temperature (T_m). The melting temperature is defined as the temperature at which a (short) piece of dsDNA is half dissociated into ssDNA. The experiment is most easily performed in a UV−vis spectrometer by monitoring the change in the absorption at 260 nm with temperature. There is a lower absorption for dsDNA than ssDNA at this wavelength, as a consequence of duplex formation. In a well-behaved duplex, a sigmoidal shape of the absorption versus temperature curve is observed because duplex scission and formation is a highly cooperative process.[425] The melting temperature is easily extracted from this curve (UV−T_m) and gives an indication about the stability of the duplex. This stability is usually lowered by introducing a mismatch into the duplex, for example, replacing a C in one strand with T, thus leading to a less stable noncanonical G−T base pair, instead of the canonical G−C.

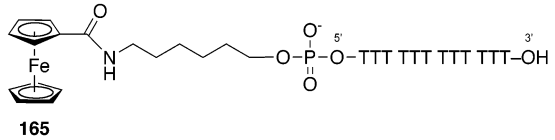
All published melting temperatures for duplex stability with ferrocenylated oligomers are summarized in Table 6. Although these data were collected by different groups under slightly different conditions, a few trends can be deduced. Substitution of the base (entries 3−9) as well as substitution at the 3′ position (entries 13 and 14) does impair base pairing significantly and leads to a marked decrease in duplex stability. It should be noted, however, that substitution at the base has been achieved by Mitsunobu reaction (entries 1 and 2) or Sonogashira coupling (entries 3−8) in all cases reported so far. With this chemistry, only T, U, and A were substituted, and thus, this conclusion is strictly true only for A−T/U base pairing. Substitution at the 2′ position (e.g. ferrocenylated RNA analogues, entries 10−12 and 15) does not seem to affect base pairing or duplex stability. If A and U Sonogashira coupling products are compared (entries 3−5 vs 6−8), the decrease in duplex stability is far more pronounced if an A is substituted with a ferrocene derivative. Finally, the Mitsunobu substitution products (entries 1 and 2) decrease duplex stability drastically in both dsDNA and DNA·RNA duplices if the ferrocene is placed in the middle of the helix. On the other hand, if ferrocene is placed close to one end of the helix, the observed effect is strongly dependent on the type of helix formed (entry 1). If a B-type helix is formed (as in dsDNA), the duplex stability is hardly influenced by the ferrocene substituent. On the other hand, in an A-type helix (as in a DNA·RNA duplex), the ferrocene substitutent is apparently less well accommodated and a marked decrease in stability is found. A very interesting case is entry 9, in that ferrocenylated U does interact much better with an opposite G than with the "correct" Watson−Crick partner A. On the other hand, in both cases, there is a comparable decrease in stability of about 5 °C as compared to the case of the perfect match with unsubstituted bases. A destabilization of similar magnitude is expected as the result of a single base mismatch.

4.3. Applications of Ferrocene-Labeled DNA Oligomers as Gene Sensors with Electrochemical Detection

The interaction, or hybridization, of two complementary oligonucleotide strands usually takes place in a strictly sequence-specific manner, that is for DNA in pairs of G−C and A−T (or A−U in the case of RNA). A so-called gene sensor capitalizes on this specific interaction. Strand I, the probe, with known sequence, interacts with strand II, the unknown oligonucleotide or sample. In an ideal world, a signal is generated if and only if strands I and II are exactly complementary. This "digital" answer is hardly ever achieved. In the real world, the kinetics of hybridization are an issue, as well as the sensitivity of the system to one or more mismatches. A variety of different signals are feasible and have been tested. The most common means of detection today is fluorescence, owing to its high sensitivity and ease of handling both chemically as a label to DNA or RNA and spectroscopically. Electrochemical detection offers an alternative means of detection which can also be highly sensitive and easy to handle. Compared to fluorescence spectroscopy, electrochemical detection is probably more robust and less prone to errors. Finally, electrochemical sensors can be built much smaller than a fluorescence spectrometer and at far lower cost. For all these reasons, there is a high interest in electrochemical DNA sensors.[397] Several reviews in recent years discussed individual aspects of electrochemical DNA sensors such as miniaturization, surfaces, and detection methods.[426−430] The high potential in this area has attracted several companies.[431,432]

In DNA or RNA oligomers, the guanine nucleobases may be electrochemically oxidized. This fact has indeed been exploited for so-called "indicatorless" electrochemical DNA hybridization sensors on carbon paste electrodes.[433] Obviously, this approach requires a guanine-free immobilized "capture strand". In addition, a relatively high potential is required and this oxidation reaction may easily be irreversible. Given the favorable electrochemical properties of ferrocene and derivatives, it is hardly surprising that labeling of oligonucleotides with ferrocene is the focus of attention in the field of electrochemical gene sensors. Ferrocene has been attached via a flexible linker to the 5′ end of a DNA oligomer, as exemplified for the ferrocenylated $(dT)_{12}$ ODN **165** in Scheme 59.

Scheme 59. Structure of a Ferrocene-Labeled $(dT)_{12}$ ODN 165



165

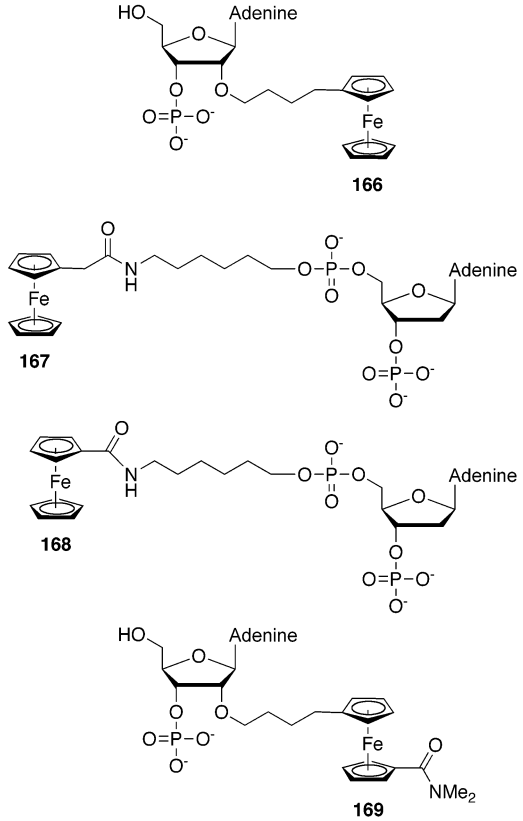
This electrochemically active probe DNA is hybridized to the complementary DNA, and the conjugate

can now be detected electrochemically, for example, by HPLC-ECD.[434−437] This technique is related to the work of Eckert et al., who used ferrocene labels to make peptides amendable to the highly sensitive electrochemical detection in HPLC.[146,203] In the HPLC-ECD system, Takenaka et al. achieved a sensitivity of down to 1 fmol of DNA under favorable conditions when a ferrocenylated T_{12}-mer was hybridized with an excess of poly(dA).[434] In a more realistic case, the same probe was hybridized with a plasmid containing a choline transporter gene (CTG) fragment of 3693 bp. The CTG promoter region contains one A_{13} sequence, and the whole CTG fragment was detected at a minimal concentration of 20 fmol. A better sensitivity was achieved using a ferrocenylated mixed sequence 20-mer as the probe,[434] and this difference was attributed to the higher melting temperature of duplices containing the longer probe. On the other hand, a higher melting temperature might imply a decreased mismatch sensitivity, but this aspect was unfortunately not investigated. The thermodynamics of triplex formation with a ferrocenylated ODN have been studied in detail.[437] A huge enthalpic gain upon triplex formation is largely canceled by entropic effects, resulting in a gain of free energy of 2−3 kcal mol^{-1}, compared to triplex formation with an unmodified ODN. A similar enthalpy−entropy compensation has been observed before in unmodified ODN triplices, suggesting that triplex formation with a ferrocenylated ODN is governed by the same major influences as with unmodified ODNs and that there is no unusual ferrocene-specific effect, at least in these systems. Finally, electrochemical data for the ferrocene moiety in the triplex are very favorable. There is only a slight sequence dependence of the redox potential of ~30 mV. Detection in this triple-helix HPLC-ECD setup is possible at the femtomole level, which compares favorably to the sensitivity of radioisotopic or enzyme-linked colorimetric assays.[437,438] In later work, the stability and structure of a 16-mer DNA triplex containing a 3-*N*-Fem-thymidine residue in the third strand have been compared with an unmodified triplex of the same sequence by differential scanning calorimetry, CD spectroscopy, and molecular modeling. In this case, the ferrocene nucleotide does not disrupt the global geometry of the triplex but lowers the apparent pK_a value of neighboring cytosines by making them more accessible to the solvent.[439]

The sensitivity of the HPLC-ECD system can be further enhanced if the sample DNA is amplified by PCR with the use of a ferrocenylated ODN primer.[440] Two DNA fragments (a 0.97 kb DNA fragment from the oncogene *v-myc* and a 0.51 kb fragment from exon 48 of the human dystrophin gene) were PCR amplified using ferrocene-modified and unmodified primers of 25 bp and 26 bp length. In both cases, correct PCR products were obtained and the efficiency of amplification with the ferrocenylated primers was about half of that of the unmodified primers. Under low numbers of PCR cycles, DNA amplification proceeds exponentially. Thus, a quantitative response was observed over at least 2 orders of magnitude, and as little as 0.1 fmol of oncogene *v-myc* DNA was detected reliably. This experiment suggests that ferrocenylated primers can be used for quantitative PCR analysis coupled with HPLC-ECD. An additional advantage of this method is the fact that no reference is required, in contrast to conventional quantitative PCR, in which the sample and a reference DNA are coamplified in one vial.

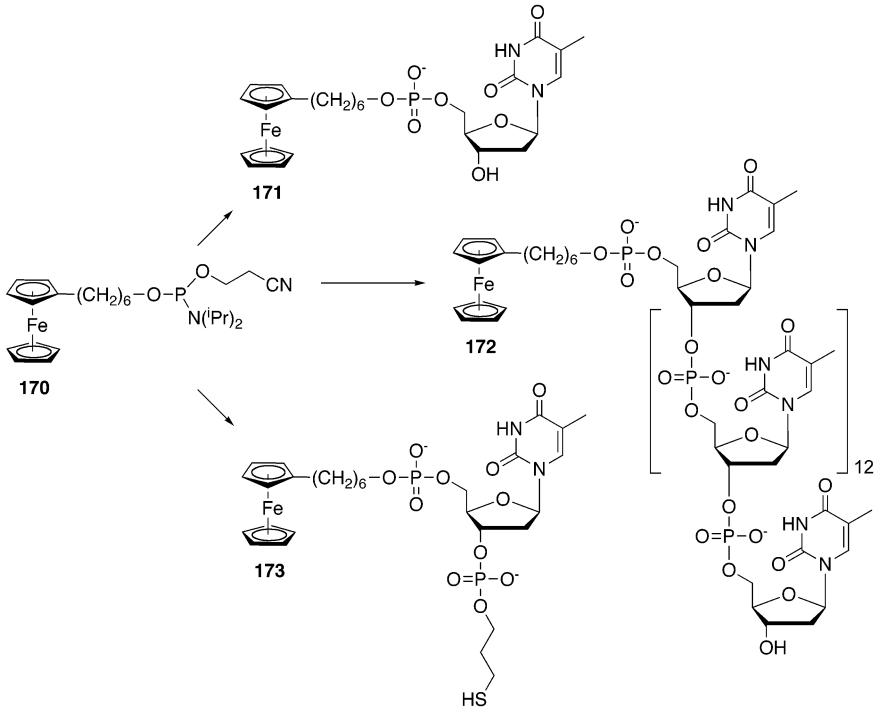
Excellent sensitivity is also achieved by coupling capillary gel electrophoresis (CGE) to electrochemical detection and using ferrocenylated ODNs.[430] This technique has been pioneered by Kuhr and coworkers.[441,442] Sinusoidal voltammetric (SV) detection was recommended for selective identification of the DNA amplification products following PCR.[441,442] This technique has also been proposed for electrochemical detetction of native amino acids and peptides.[443] A strategy for so-called "four colour DNA sequencing" has been developed by this group.[441] As an example, the T3 PCR primer was covalently 5′-modified with four different ferrocene derivatives **166**−**169** (Scheme 60). Their redox potentials span a range of 230 mV,

Scheme 60. Four Ferrocenylated A Monomers with Different Redox Potentials: $E_{1/2}$ (CV with 1 V/s, vs Ag/AgCl) = 39 mV (166), 73 mV (167), 202 mV (168), and 268 mV (169)



but the minimal difference between **166** and **167** is only 34 mV. This difference, which would be difficult to differentiate reliably in a normal CV, is readily discernible by SV. When coupled to CGE separation, the discriminating power of the technique was shown to be applicable to "low-resolution" DNA sequencing.[441] By combining the single base PCR extension technique of a ferrocenylated primer with CGE and electrochemical detection, a novel system for single nucleotide polymorphism (SNP) analysis was estab-

Scheme 61. A 5′-Ferrocenylated Phosphoramidite (170), a 5′-Ferrocenylated Thymine Monomer (171), a 5′-Ferrocenylated $(dT)_{14}$ ODN (172), and a 5′-Ferrocenylated Thymine Monomer with a 3′-Thiol Group for Immobilization on a Au Surface (173)



lished. The unextended 20-mer primer could be separated from the 21-mer extension product, thus demonstrating a single-base resolution separation of a DNA oligomer with electrochemical detection.[442]

The development of chip technology has revolutionized genetic analysis. On one chip, a large number of ODNs with different sequences (typically thousands, but chip densities up to millions of ODN per cm^2 have been achieved) are immobilized in a spatially addressable manner. Complementary DNA or RNA sequences from a sample bind to these ODNs, and a signal is generated at an exactly defined place. Again, fluorescence spectroscopy is the main technique for the readout of this signal. The detection of hybridization events in a chip format in combination with electrochemical techniques is particularly elegant because the surface of an electrode may directly serve as the chip on which ODNs are immobilized. Clearly, no further equipment (like a laser and CCD camera for fluorescence excitation and detection) is required.

Letsinger et al. were the first to immobilize ferrocenylated ODNs in a self-assembled redox-active monolayer on a Au surface.[444] 6-Hydroxyhexylferrocene was reacted with β-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to yield the corresponding ferrocenyl amidite **170**, which was coupled to thymidine modified CPG. In addition to the monomer **171**, a 5′-ferrocenylated $(dT)_{14}$ ODN **172** was synthesized, as well as a ferrocenylated thymidine monomer with a 3′-thiol group (**173**, Scheme 61). Compound **173** was adsorbed on the surface of Au electrodes as a monolayer. The CV of an Au substrate modified in this manner exhibited a reversible wave at slightly more positive potential than was observed for **171** in solution. In addition to applications as electrochemical DNA sensors, such self-assembled DNA monolayers with electrochemically active groups may provide information on the mechanism of electron transfer through DNA as well as on the flexibility of short DNA. Kraatz and co-workers used differences in the ferrocene redox potential and k_{ET} values to differentiate between different modes of electron transfer between a Au electrode and ferrocene-labeled dsDNA, in particular intrastrand ET versus interstrand crossing mechanisms.[445] In a later paper, the properties of ferrocene-labeled ssDNA adsorbed on Au electrodes were studied.[446] When ssDNA with a ferrocenyl probe is bound to an Au electrode, it may be expected to adopt an unordered coil conformation. If, on the other hand, a complementary ODN is added, the coil will open up and the DNA double helix will form. These conformational changes result in changes in the mobility of the ferrocenyl reporter group, which in turn changes its electron-transfer properties, as shown by Anne et al. in a very interesting recent publication.[447] In fact, this concept is similar to the so-called molecular beacons or "light-up" probes, in which the fluorescence from a probe is switched off by a nearby quencher. Upon binding of the complementary ODN, probe and quencher are spatially separated and a fluorescence signal can be picked up. This principle has been translated into a DNA detection system in which the probe is an immobilized, ferrocene-labeled ODN.[448] Upon binding of a complementary ODN, the distance between the ferrocene and the Au surface is significantly altered and the peak current decreases. This system shows a remarkable linear range over 5 orders of magnitude with target DNA concentrations as low as 10 pM. In contrast to the molecular beacons with fluorescent detection described above, this is rather a "light-off"

device. Unfortunately, such systems are more susceptible to false-positive responses. In addition, picomolar sensitivity is in most cases not enough for direct detection of pathogen DNA without amplification. This limitation may be overcome by PCR amplification, in addition to the use of labels with different redox potentials, as discussed above, that will allow for differentiation between real-positive and spurious signals. As exemplified by these very first reports in this field, there is clearly a high potential for synthetic organometallic chemistry combined with techniques commonly used in molecular biology. Another recent example for a "light-off" probe demonstrates that further improvements in handling of electrochemical DNA detection are possible by using double-stranded probes and a competitive hybridization assay.[449] In this case, very good sensitivity (subnanomolar) and mismatch sensitivity is achieved by liberating a ferrocenylated DNA strand from the electrode surface without the need for additional washing steps.

A slightly different approach to an immobilized electrochemical gene sensor was published by Ihara and co-workers, using a three-ODN system,[450] later termed "sandwich assay" (Figure 12).[436] First, an

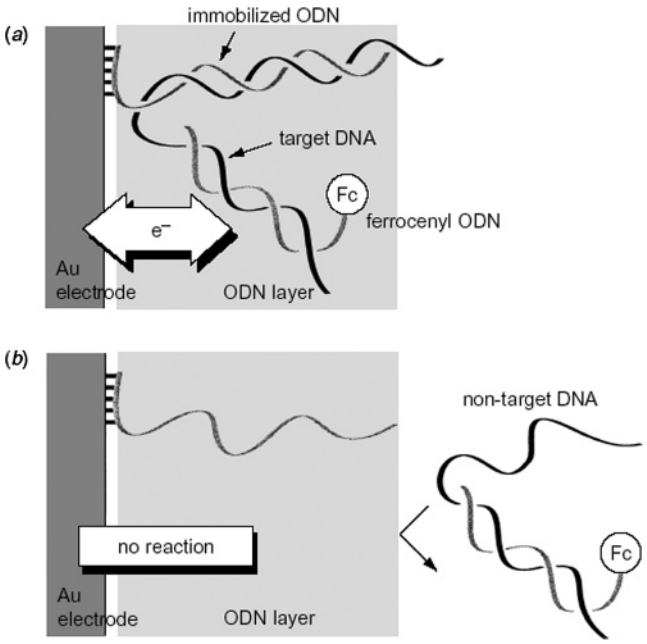
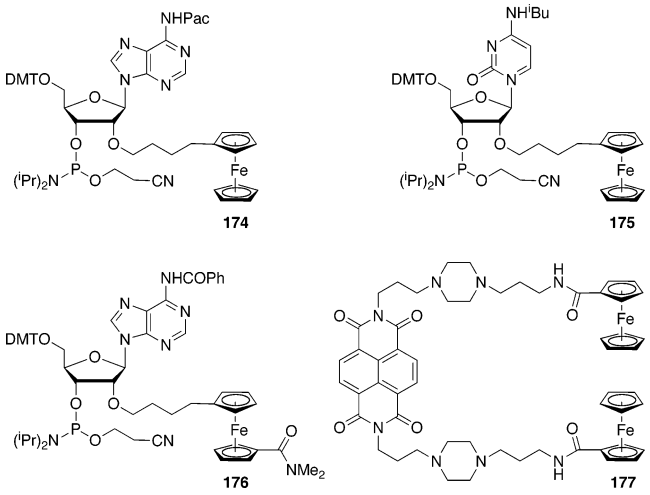


Figure 12. Principle of the "sandwich assay" for electrochemical DNA sensors. Reprinted with permission from ref 450. Copyright 1997 The Royal Society of Chemistry.

ODN capture probe is immobilized on an Au electrode surface, in this case by five successive phosphorothioate units. The target DNA binds to the immobilized capture probe if the complementary sequence is present. In addition, the target DNA carries a binding sequence (here a $(dA)_{12}$) for the ferrocenylated signaling probe DNA **165**. The ferrocenylated $(dT)_{12}$ probe **165** hybridizes with the poly-A sequence of the target DNA. If the target DNA also binds to the immobilized capture probe ODN, the ferrocene is brought close to the electrode and a significant anodic peak due to oxidation of the ferocene is observed in the DPV of the system. If G is replaced with C, generating a single CC mismatch in the target DNA sequence, binding to the immobilized probe becomes much weaker. As a result, fewer ferrocene molecules are held in the vicinity of the electrode, and consequently, only a very small anodic peak was observed in the DPV.[450] This system was later characterized in detail, including a thorough analysis of the ODN-modified surface by IR spectroscopy and a quartz crystal microbalance (QCM) study.[451]

This detection scheme was more recently refined by Yu and co-workers. In contrast to most other groups, who used 5′-terminal substitution of nucleotides with ferrocene, these workers synthesized 2′-ferrocenyl nucleotides such as **174** and **175** (Scheme 62).[452] These compounds are the first ferrocenyl-RNA

Scheme 62. Three Electronically Active 2′-Modified Phosphoramidite Nucleotides 174−176 and an Electrochemically Active Threading Intercalator 177 Proposed by Takenaka



derivatives, and as such, they are more versatile chemically because they may be incorporated into an oligonucleotide strand at *any* position and even ODNs with multiple ferrocenyl incorporation are feasible. Whereas the synthesis of the adenosine derivative **174** was straightforward and the corresponding phosphoramidite could be obtained in reasonable yield, the cytidine derivative **175** was obtained together with the 3′-ferrocenylated isomer in a 2:1 ratio. However, both isomers could be separated on silica in the form of their 5′-DMT derivatives. These protected phosphoramidites can be directly used in solid-phase DNA synthesis. In thermal melting studies of mixed-sequence 15-mer ODNs, replacement of either A with **174** or C with **175** did not produce a significant effect on the melting temperature of the duplices. However, if the 2′-ferrocenylated **175** was replaced with its 3′ isomer, the melting temperature of the duplices decreased by ~4 °C, comparable to introduction of a single GG mismatch at the same position. The CV of a 15-mer ODN containing **174** showed a reversible wave virtually at the same position as that for a water-soluble ethylferrocene derivative under the same experimental conditions. Recently, the development of a versatile platform for molecular diagnostics on microarrays based on this chemistry was described in more detail.[453] A sandwich assay format

was used in which the DNA capture probe was immobilized in a self-assembled monolayer (SAM) on a gold electrode. Electrochemically active signaling probes containing 2′-ferrocenylated adenosine were employed and held in close proximity of the SAM upon concomitant binding to the unlabeled target DNA. The system has been successfully tested for sequence-specific electrochemical detection of PCR-amplified DNA without the need for further purification. The usefulness was further demonstrated by SNP analysis and gene expression monitoring with these simple and relatively inexpensive microarray systems.[453] Also, an application for the screening for point mutations in a gene associated with hereditary hemochromatosis has been described.[432]

To alter the electrochemical properties of DNA oligomers in dependence of the DNA sequence, a second A monomer **176** with a different ferrocene derivative attached to the 2′ position was prepared (Scheme 62).[454] The difference of the redox potentials of **174** and **176** is 170 mV as a consequence of the dimethyl carboxamide group in the latter. Sequence-dependent electrochemical detection now works as follows. A capture probe (23-mer ODN) is immobilized on a Au surface. The target ODN is known to contain the sequence complementary to the capture probe and binds with high affinity. Finally, the signaling probe binds to another part of the target ODN. Two different 16-mer ODN signaling probes **I** and **II** were used which are extended with three molecules of **174** or **176**, respectively, at the 5′ end. In addition, both signaling probes differ in sequence only in one base. Depending on the exact sequence of the target DNA, one probe will bind preferentially, bringing different ferrocene derivatives with different redox potentials close to the surface. This difference is easily detected by alternating current voltammetry (ACV). This scheme uses similar ferrocene probes to those of the work of Kuhr discussed above and clearly holds a lot of promise, although the difference in stability (melting temperature) was not reported, nor was any further mismatch sensitivity investigated.

In a recent interesting publication, the extension of such detection systems to RNA has been described.[455] The electrochemically active RNA monomer **164c** (Scheme 58) was synthesized and fully characterized. **164c** could be incorporated into RNA oligomers in place of U by two different RNA polymerases. Increasing the **164c**/U ratio indeed produced more heavily labeled transcripts, as shown by gel electrophoresis and an increased area-under-the-curve in SWV of the RNA transcripts. These ferrocene-labeled RNA oligomers could be immobilized on Au electrodes (SAM), and the system was successfully used for the electrochemical detection of very small amounts of RNA. An electrochemical "two-colour" assay is proposed by using **164c**-modified RNA oligomers along with anthraquinone-labeled UTP monomers.[455]

Another interesting aspect is the detection of DNA damage by hybridization of a ferrocenylated probe ODN with immobilized ODNs on a graphite electrode.[456] The amount of hybridized probe DNA is determined as the anodic peak current of ferrocene by differential pulse voltammetry (DPV). If the sample DNA is deliberately damaged, for example, by the action of hydroxyl radicals, less probe DNA binds and a smaller signal is detected. This experimental setup was then used to assess the protection efficiency of various hydroxyl radical scavengers, and the results were found to correlate well with the literature. In their work on sequence-specific DNA detection, Fang et al. used the same linker as Takenaka for covalent attachment of ferrocene to the 5′ end of DNA oligomers. However, ferrocene was attached via an imine bond rather than as the amide.[457] As an alternative to Au or graphite electrodes, Fang's group has proposed the use of glassy carbon electrodes which were coated with a chitosan oligomer film.[458] This positively charged polymer forms very stable complexes with negatively charged DNA. Still the DNA hybridizes with a complementary ODN probe, which carries a ferrocene label, leading to an electrochemical signal. A linear relation of the anodic peak current to the amount of immobilized DNA over more than 2 orders of magnitude was measured. Interestingly, this paper also reports the reaction of the primary amino group of an aminoferrocene derivative with the terminal phosphate group of an ODN after activation with imidazole and EDC.[458] For future applications, the linker may be improved toward better electron-transfer properties, as shown by Creager and co-workers.[459] Also, silicon may turn out to be an attractive alternative to Au also for electrochemical DNA devices.[460] For instance, Si/SiO_2 surfaces can be modified with covalently linked oligonucleotides via amino- or glycidoxysilane linkers.[461,462] The hybridization event may be measured by changes in the impedance. A direct readout of the T_m value for dsDNA is also possible with such a device.[463] Wayner and co-workers have published work on the covalent modification of Si surfaces by direct silicon−carbon bond formation.[464,465]

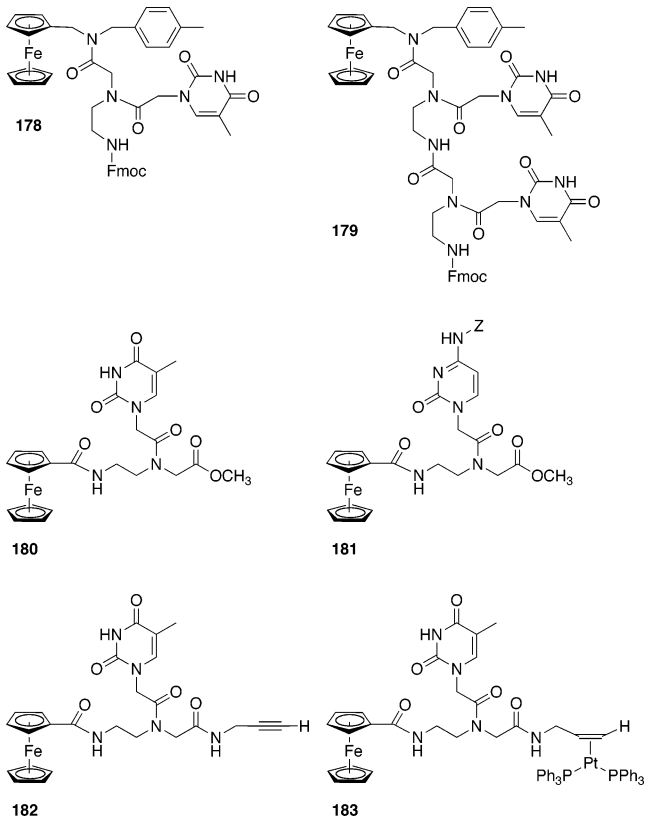
Finally, attempts have been made to do electrochemical DNA detection without having to use metal-modified ODNs.[436] This can be achieved by immobilizing the capture ODN on an electrode, allowing it to hybridize with the target DNA, and then adding an electrocactive compound that will interact preferentially with dsDNA. Quite a number of compounds have been used for detection, including Co complexes, ethidium bromide, and intercalating drugs such as daunorubicin. Takenaka's group has successfully used ferrocene-modified naphthalenediimide (**177**) as a threading intercalator (Scheme 62).[466−468] This system is chemically very robust and highly sensitive, reaching a detection limit of 10 zmol DNA with DPV.[466] The sensitivity of the system could be enhanced by coupling it to an enzymatic reaction like the glucose oxidation using glucose oxidase (GOD).[468] Furthermore, the system has the potential of direct mismatch detection by determining the number of intercalated molecules or the rate of electron transfer.[467] In a perfect match situation, one molecule of **177** is intercalated between every second base. It is assumed that this number will decrease if the local geometry is perturbed by a single base mismatch and less electrons will be transferred less fast as a

consequence. Evidently, this system needs careful calibration and probably optimization for every single application. On the other hand, it is versatile in the sense that one and the same simple electroactive probe (**177**) is used for every ODN sequence to be investigated, making the synthesis of ferrocene-labeled ODNs—with all maybe unexpected problems—unnecessary. An application that enables the rapid analysis of heterozygous deficiency of the human lipoprotein lipase gene has been reported.[469]

4.4. Ferrocene Derivatives of PNA Monomers and Oligomers

Peptide nucleic acids (PNAs) are a class of DNA analogues in which the ribose phosphate ester backbone is replaced by a pseudo-peptide backbone.[470−472] The nucleobases are linked to this backbone via a carboxymethylene linker.[473,474] PNAs bind to complementary DNA or RNA oligomers according to Watson−Crick rules with high stability[475−480] and have found applications in molecular biology and as antisense agents.[471,472,481] The Metzler-Nolte group has prepared the first organometallic derivatives of PNA monomers. The ferroceneamine derivative **81a** has been used for the reaction with the C-terminus of a number of amino acid and peptide derivatives (see section 2.3.2). Similarly, the T-PNA ferrocene derivative **178** (Scheme 63) has been prepared. The *N*-Fmoc

Scheme 63. Ferrocene Derivatives of PNA



protecting group can be readily removed under mild conditions, and coupling to a second T-PNA monomer is possible, yielding the ferrocenylated PNA dimer **179**. Ferrocenecarboxylic acid (**40**) could also be coupled to the amino group of PNA monomers using HBTU as the coupling agent to give compounds **180** (T) and **181** (Z-protected C).[139] The activation barrier $\Delta G^{\ddagger}$ for the rotation about the tertiary amide bond has been determined for the ferrocenylated T-PNA monomer **180** to be 75 ± 0.5 kJ mol^{-1}. For **180**, a T−T self-association constant $K_{TT} = 2.5 \pm 0.2$ M^{-1} has been measured by ^{1}H NMR dilution experiments in $CDCl_3$. In comparison, the association constant k_{AT} for the regular A−T Watson−Crick base pair was found to be 78 ± 9 M^{-1} (in dry $CDCl_3$) by the same experimental technique, using **180** and tris(isopropyl)adenosine as a soluble adenine derivative. These values are also the only association constants for PNA monomers in the literature. Within the experimental limits, these values correspond well to literature values, indicating (a) that there is no additional stabilization or destabilization to base pairing of the PNA amide backbone and (b) that Watson−Crick base pairing is not impaired by the metallocene at the N-terminus.

The first bimetallic derivative of PNA monomers was prepared in the Metzler-Nolte group in the form of **183**. This compound was prepared by coordination of the $Pt(PPh_3)_2$ fragment to the ferrocenyl T-PNA alkyne **182**. Like all PNA monomers and single-stranded oligomers, compound **183** exists as a mixture of *cis-/trans*-isomers at the tertiary amide bond. In addition, it shows characteristic NMR signals as a consequence of coupling to two inequivalent ^{31}P nuclei and the Pt nucleus. Finally, ferrocene was also incorporated into PNA oligomers by solid-phase peptide synthesis.[482] The PNA heptamer Fmoc-tggatcg-Gly was prepared on solid support by standard Fmoc PNA synthesis methods. After deprotection of the last Fmoc group, the heptamer was reacted with activated ferrocenecarboxylic acid *on the resin*. Cleavage from the resin was achieved by methanolic ammonia with simultaneous removal of the exocyclic protecting groups. Only one main product was observed in reverse phase HPLC of the crude reaction product (>90%). The conjugate Fc-CO-tggatcg-Gly-NH_2 (**184**) was purified by preparative HPLC and shown to have the correct mass by MS. Compared to a tris(bipyridyl)Ru and an acetyl derivative of the same sequence, **184** was by far the least hydrophilic derivative and consequently not very soluble in aqueous solvents.

5. Conjugates of Ferrocene with Carbohydrates

With only very few exceptions, all ferrocene sugar derivatives reported so far are either ferrocenylmethyl derivatives or esters, thioesters, or amides obtained from ferrocenecarboxylic acid. 1,1′-Disubstituted ferrocene derivatives are almost exclusively derivatives of ferrocene-1,1′-dicarboxylic acid. Typical reactions were the same as those for derivatives described in previous sections, that is, alkylation with trimethylferrocenylmethylammonium iodide (**154**), Schiff base formation with ferrocenecarbaldehyde (**67**) followed by reduction to the amine, and reaction with activated ferrocene(di)carboxylic acid.

5.1. Carbohydrate Derivatives of Monosubstituted Ferrocene

Table 7 summarizes most of the simple monosubstituted ferrocene derivatives described so far. The

Table 7. Carbohydrate Derivatives of Ferrocene

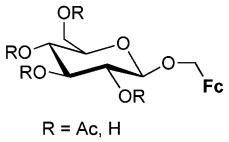
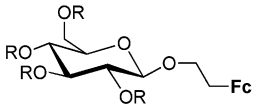
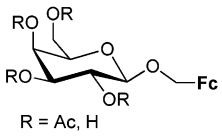
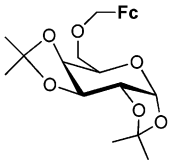
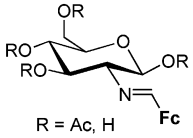
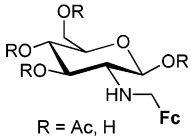
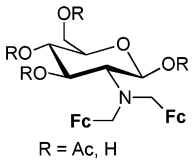
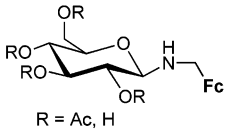
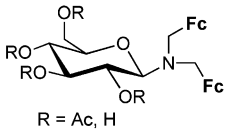
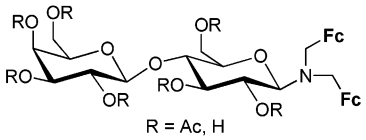
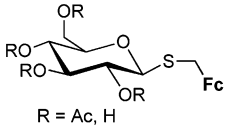
classification	structure		sugar derivative	ref
O-alkylated	 R = Ac, H	**185a**: R = Ac **185b**: R = H	β-D-glucopyranose	483, 484
		**186**: R = H	β-D-glucopyranose	483
	 R = Ac, H	**187a**: R = Ac **187b**: R = H	β-D-galactopyranose	485
		**188**	α-D-galactopyranose	487
N-alkylated	 R = Ac, H	**189a**: R = Ac **189b**: R = H	β-D-glucosamide	487 (**189b** only)
	 R = Ac, H	**190a**: R = Ac **190b**: R = H	β-D-glucosamide	489, 487 (**190a** only)
	 R = Ac, H	**191a**: R = Ac **191b**: R = H	β-D-glucosamide	489, 487 (**191a** only)
	 R = Ac, H	**192a**: R = Ac **192b**: R = H	β-D-glucopyranosylamine	489
	 R = Ac, H	**193a**: R = Ac **193b**: R = H	β-D-glucopyranosylamine	489
	 R = Ac, H	**194a**: R = Ac **194b**: R = H	β-D-galacto-β-D-glucopyranosylamine	489
S-alkylated	 R = Ac, H	**195a**: R = Ac **195b**: R = H	β-D-thioglucopyranose	487, 486 (**195b** only)

Table 7 (Continued)

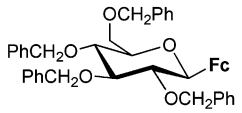
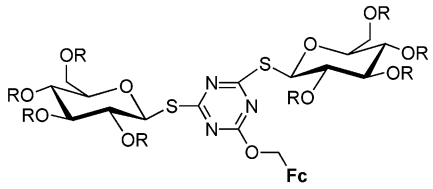
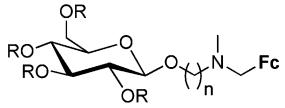
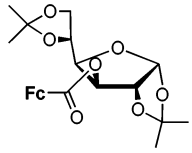
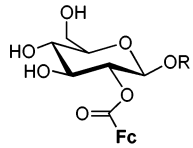
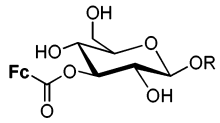
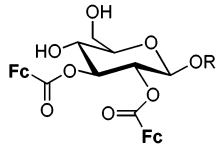
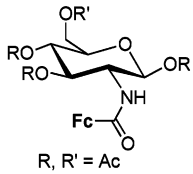
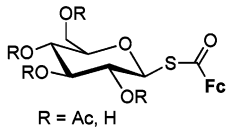
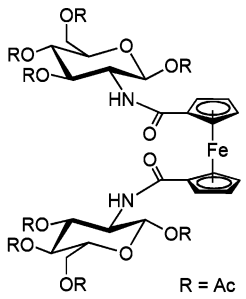
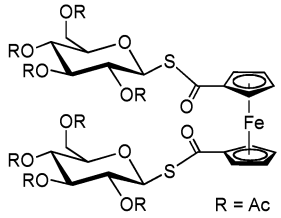
classification	structure		sugar derivative	ref
anomeric C substituted		**196**	deoxy-β-D-glucopyranose	491
Others		**197**	β-D-thioglucopyranose (twice)	486, 487
	 n = 2, 3, 5 R = CH_2Ph (**a**), H (**b**), Ac (**c**)	**198**: n = 2 **199**: n = 3 **200**: n = 5 **a**: R = CH_2Ph, **b**: R = H, **c**: R = Ac (only for **198**)	β-D-glucopyranose	488
Ester		**201**	α-D-glucofuranose	487
		**202**	β-D-glucopyranose	498
		**203**	β-D-glucopyranose	498
		**204**	β-D-glucopyranose	498
Amide	 R, R' = Ac R, R' = H R = H, R' = PO_3H	**205a**: R = R' = Ac **205b**: R = R' = H **206**: R = H, R' = PO_3H	β-D-glucosamide	487 (**205a**), 493 (**205b** and **206**)
Thioester	 R = Ac, H	**207a**: R = Ac **207b**: R = H	β-D-thioglucopyranose	486, 487, 492
1,1'-Diamide	 R = Ac	**208**: R = Ac	β-D-glucosamide (twice)	487

Table 7 (Continued)

classification	structure		sugar derivative	ref
1,1'-Thioester		**209**: R = Ac	β-D-thioglucopyranose (twice)	486, 487, 492

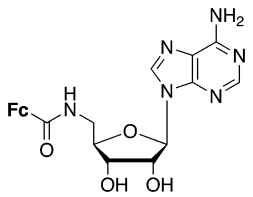
very first sugar derivatives of ferrocene were reported in 1961,[483] only one decade after the discovery of ferrocene itself and four years after the first report on ferrocenylamino acid derivatives. Compound **185b** was prepared from hydroxymethylferrocene and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide in the presence of AgO and $CaSO_4$, followed by base hydrolysis of the acetyl protecting groups. This compound was used in a kinetic investigation of hydrolysis of the glucosidic bond and compared to **186**, which does not yield the relatively stable ferrocenylmethyl cation.[407,483] However, the results of this study were later corrected.[484] Similarly, kinetic results from hydrolysis of the galactose derivative **187b** helped to shed light on the mechanism of the enzyme *β*-galactosidase.[485]

In a comprehensive preparative study, Adam and Hall reported the synthesis of a wide variety of ferrocene sugar derivatives by different methods.[486,487] Glucosamine derivatives **190a** were obtained by Schiff base formation of **189a** with ferrocenecarbaldehyde (**67**),[487] followed by reduction.[488] From this reaction, the *N*,*N*-bis(Fem) derivative **191a** could also be isolated.[488] The formation of **191a** parallels the isolation of $(Fem)_2$Gly-OMe (**76**) mentioned in section 2.3.2. The same compounds were also readily prepared by reaction of glucosamine with ferrocenylmethyl tosylate.[487] Indeed, this reagent proved to be superior to trimethylferrocenylmethylammonium iodide (**154**) in that higher yields of the desired products were obtained under milder conditions. For example, the thiosugar derivative **195a** was obtained in 2 days at room temperature in enantiomerically pure form, whereas reaction with **154** required reflux in acetonitrile with anhydrous Na_2CO_3, leading to isomerization at the anomeric carbon atom (α/β = 2:3). The glucopyranosylamine derivatives **192a**−**194a** were obtained by reaction of glucopyranosylamine with **154** under retention of the stereochemistry at the anomeric carbon atom.[489] Careful hydrolysis yielded the unprotected sugars **192b**−**194b**. Compounds **190a** and **191a** could be deacetylated to yield **190b** and **191b** by treatment with Amberlite IRA 400 (OH) resin in methanol. In their earlier study, Adam and Hall were unable to obtain the deprotected derivatives without decomposition.[487] Only the relatively stable thiosugar derivative **195b** could previously be deacetylated with NaOMe in methanol. The preparation of three imino sugar derivatives from ferrocene was also reported by Schneider and Wenzel in 1979 (of glucosamine, galactosamine, and mannosamine).[490] Catalytic hydrogenation in ethanol in the presence of PtO_2 reduced not only the C=N double bond but also the amino sugar aldehyde group, yielding ferrocenylmethyl derivatives of amino sorbit, amino dulcit, and amino mannit. However, no further characterization apart from MS data was reported.[490]

Another class of water-soluble ferrocenecarbohydrate conjugates was reported recently by Robinson and co-workers. They prepared benzyl derivatives **198a**−**200a**, which could be deprotected by careful catalytic hydrogenation, yielding the fully unprotected **198b**−**200b**.[488] Compound **198b** was also prepared by a simpler route via the acetylated derivative **198c**, which was successfully deacetylated using the IRA 400 (OH) resin. The anomerically pure *β*-D-glucopyranosyl derivative **196** was obtained in a C−C bond-forming reaction in 31% yield via the Friedel−Crafts alkylation reaction from an anomeric mixture (α/β = 4:1) of benzyl-protected 1-*O*-acetyl-D-glucopyranose.[491] In the same reaction, thiophene and benzene gave yields of 62% and 23%, respectively, as would be predicted from simple considerations of electron density of the aromatic ring.

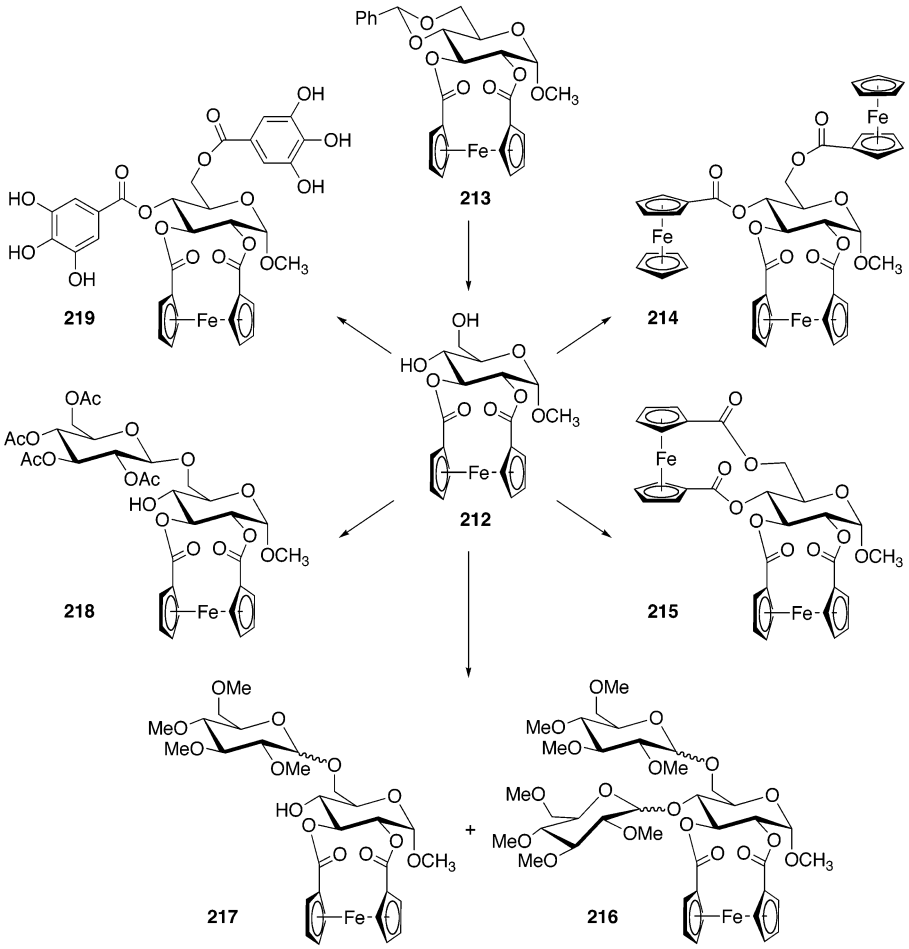
Most acetylated derivatives listed in Table 7 were reported by Adam and Hall (**201**, **205a**, **207**−**209**; but see also chapter 5.2 for ester derivatives of ferrocene-1,1′-dicarboxylic acid).[486,487,492] In all preparations, ferrocene(di)carboxylic acid chloride was used and ferrocenecarboxylic acid anhydride was frequently observed as a byproduct. Only the deprotected **207b** could be obtained without decomposition. In a recent paper, Kraatz et al. reported the synthesis of **205b** and **206** directly from the activated ferrocenecarboxylic acid benzotriazol (**43**) and the unprotected glucosamine and glucosamido-2′-phosphate. This paper also reports the synthesis of 5′-ferrocenoylamidoadenosine (**210**); see Scheme 64.[493] These water-soluble ferrocene derivatives were subjected to electrochemical studies in aqueous solution at different pH values. The oxidation of **205b** is fully reversible between pH 2 and pH 9. At pH 12,

Scheme 64. 5′-Ferrocenylamidoadenosine 210



210

Scheme 65. Synthesis of 2,3-(Ferrocene-1,1′-dicarbonyl)-*O*-α-D-glucopyranoside (212) and Derivatives Thereof



however, the oxidation becomes irreversible and a mechanism of decomposition is proposed.

5.2. Carbohydrate Derivatives of Ferrocene-1,1′-dicarboxylic Acid

5.2.1. Esters

2,3-(Ferrocene-1,1′-dicarbonyl)-*O*-α-D-glucopyranoside (**212**) has been the key starting material for a number of derivatives, as summarized in Scheme 65. Applications as chiral matrixes were evaluated by

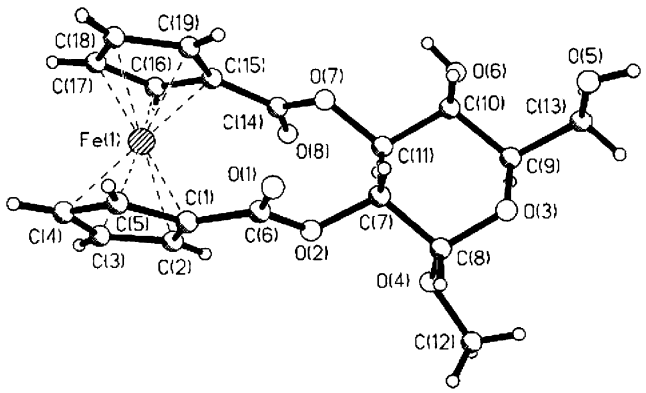
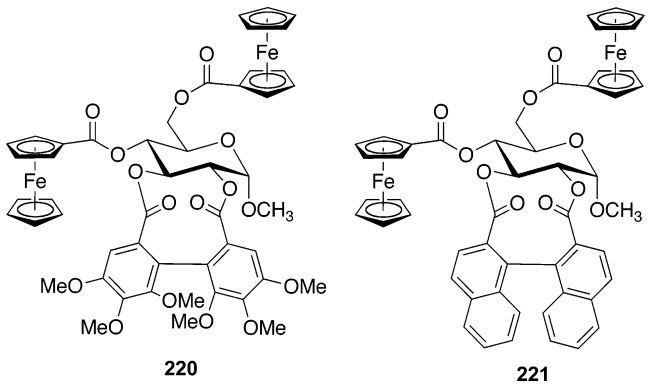


Figure 13. X-ray single-crystal structure of **212**. Reprinted from ref 499, by permission of The Royal Society of Chemistry (RSC) on behalf of the Centre National de la Recherche Scientifique (CNRS). Copyright 2002.

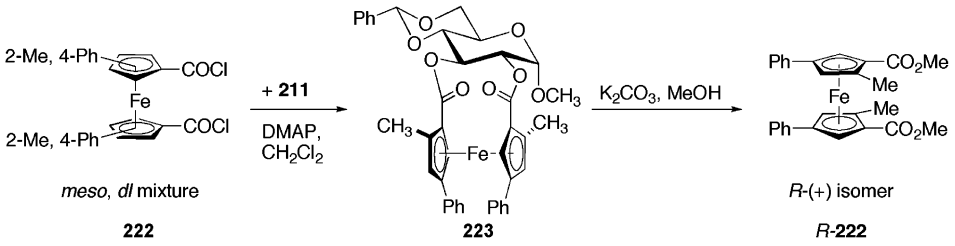
Scheme 66. Ferrocenylated Biphenyl (220) and Binaphthyl (221) Ellagitannin Derivatives



Itoh and co-workers,[494−496] and medicinal applications were proposed by the groups of Itoh[497,498] and Keppler.[499]

Compound **212** is readily prepared by reaction of ferrocene-1,1′-dicarboxylic acid chloride (**63**) with 4,6-benzylidene-*O*-methyl-α-D-glucopyranoside (**211**) to yield **213**, followed by benzylidene deprotection (Scheme 65).[497,499] The solid-state structure of this compound is shown in Figure 13. Reaction of **212** with ferrocene(di)carboxylic acid chloride (**41** or **63**) yields **214**[498] and **215**,[499] respectively. From **212**, the disaccharides **217** and **218** and the trisaccharide **216** were obtained in about 50% yield.[498] Itoh's group has

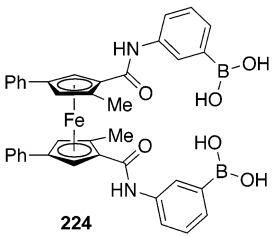
Scheme 67. Chiral Resolution of Planar Chiral Ferrocene Derivatives via the Sugar Intermediate 223



also prepared ferrocenoyl derivatives **219**−**221** of ellagitannins (Scheme 66),[497] which constitute the main curative and palliative ingredient in various traditional herbal medicines. The chiral biphenyl derivative **220** as well as the binaphthyl derivative **221** were actually synthesized in enantiomerically pure form.[497,498] All ferrocene derivatives **212**−**221** as well as **202**−**204** were tested for their antimalarial activity against a chloroquine-sensitive *P. falciparum* strain.[498] All compounds were far less active than quinine, and only (SD)-**220** showed an EC_{50} value in the high nanomolar range. Surprisingly, all compounds showed similar antitumor activity against a mouse mammary tumor cell line with rather high EC_{50} values (around 20 μM). Keppler and co-workers later found even worse cytotoxic activity for **212** in four other tumor cell lines.[499]

In another interesting application, Itoh's group has been able to resolve the racemic mixture of the planar chiral ferrocene **222** into enantiomerically pure *R*-**222** via the sugar intermediate **223** (Scheme 67).[494] The absolute configuration of **222** was established by a combination of computational methods, CD spectroscopy, and ^{1}H NOE NMR spectra of the sugar intermediate **223**. Bearing in mind that the protected sugar **211** is inexpensive and readily available, this procedure provides a straightforward way to enantiomerically pure planar chiral metallocenes. Enantiomerically pure *R*-**222** has subsequently been turned into a diboronic acid derivative **224**, which was used for complexing a variety of different sugars (Scheme 68).[495] Binding of different sugars with different

Scheme 68. Enantiomerically Pure Ferrocene Diboronic Acid Derivative 224 for the Complexation of Sugars

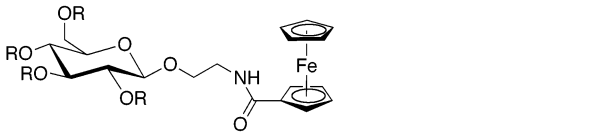


absolute chirality was monitored by UV−vis and CD spectroscopy. Although marked changes were observed upon binding, it was unfortunately not possible to derive simple rules to predict or explain those spectral changes. On the other hand, preferential binding for some sugars could be rationalized by steric crowding using molecular modeling.[496]

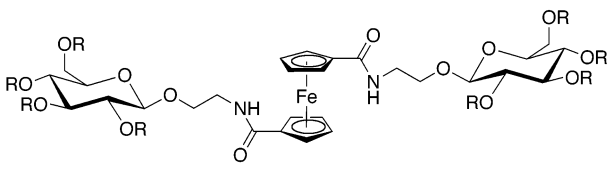
5.2.2. Dendrimers

Credi and co-workers have recently published an extensive study on ferrocene-containing carbohydrate dendrimers.[500] Compounds **225**−**228** (Scheme 69)

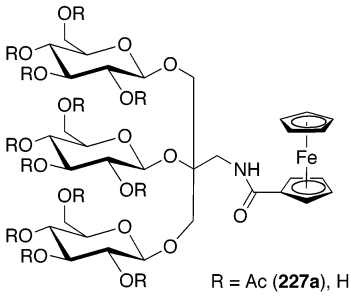
Scheme 69. Ferrocene Carbohydrate Dendrimers



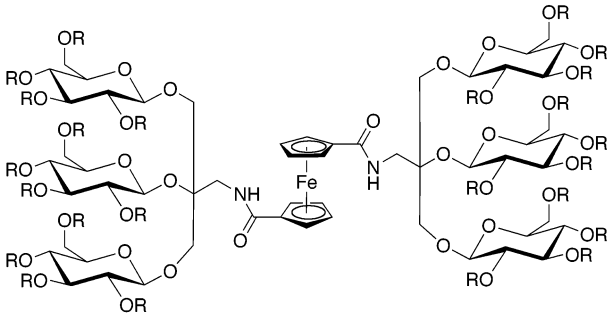
R = Bz (**225a**), H (**225b**)



R = Bz (**226a**), H (**226b**)



R = Ac (**227a**), H (**227b**)



R = Ac (**228a**), H (**228b**)

were prepared by coupling of the ferrocene acid chlorides to the amino group of the protected sugars. Deprotection of the benzyl or acetyl derivatives **a** was claimed to be quantitative. Electron-transfer reactions of the ferrocene core were thouroughly investigated for all compounds. The same trends as shown for many other ferrocene carboxylic acid derivatives prevail. The potential of the reversible one-electron oxidation of ferrocene is about 200 mV more positive for the disubstituted derivatives **226** and **228** com-

pared to **225** and **227**. On the other hand, potentials for the deprotected compounds **b**, which were determined in water, were consistently about 200 mV more positive than those for protected derivatives **a** (measured in acetonitrile). This finding was attributed to a better stabilization of the ferrocenium ion in the more polar aqueous medium. The disubstituted dendrimeric **228** showed a peculiar electrochemical behavior which suggests the presence of at least two species in solution, possibly a *cisoid* and *transoid* form of the substitutents on the two Cp rings. The luminescence quenching of excited $[Ru(bpy)_3]^{2+}$ is faster than the diffusion rate constant for the solvent. This leads to the conclusion that the rate-limiting step of the quenching process is that within the encounter complex. The observed increase in the diffusion-corrected quenching rate k_{cor} for the larger derivatives **227** and **228** corresponds to the increased shielding of the ferrocene moiety and is in agreement with the Dexter mechanism of double electron exchange.[501] Only the monosubstituted derivatives **225b** and **227b** were complexed by β-cyclodextrin (β-CD) in aqueous solution with stability constants k_{stab} of 2000 ± 200 L mol^{-1} (**225b**) and 1300 ± 200 L mol^{-1} (**227b**), respectively. These values were determined electrochemically. A shift to more positive potential for the one-electron oxidation of ferrocene upon addition of β-CD was readily explained by the fact that the complexed ferrocene derivatives are electrochemically inactive and, therefore, the electron-transfer process must be preceded by decomplexation from β-CD (Figure 14).[502,503]

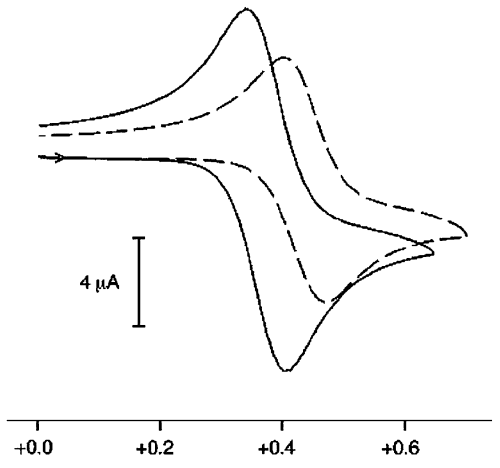


Figure 14. Cyclic voltammogram of **225b** in the absence (full line) and presence (dashed line) of 10 equiv of β-CD. 1 mM solution in H_2O, 0.1 M $NaClO_4$, glassy carbon electrode, 100 mV/s. Reprinted with permission from ref 500. Copyright 2002 Wiley-VCH.

5.3. Cyclodextrins

Cyclodextrins be may regarded as sugar derivatives and have been studied in their interaction with ferrocene. In ethylene glycol, ferrocene alone was shown to have an axial position in β-CD, whereas an equatorial position was assumed in the larger cavity of γ-CD, as was suggested by a positive induced CD band around 450 nm for the inclusion complex Fc-β-CD and a similar but negative CD band for Fc-γ-CD (Figure 15).[504] More details, including stability

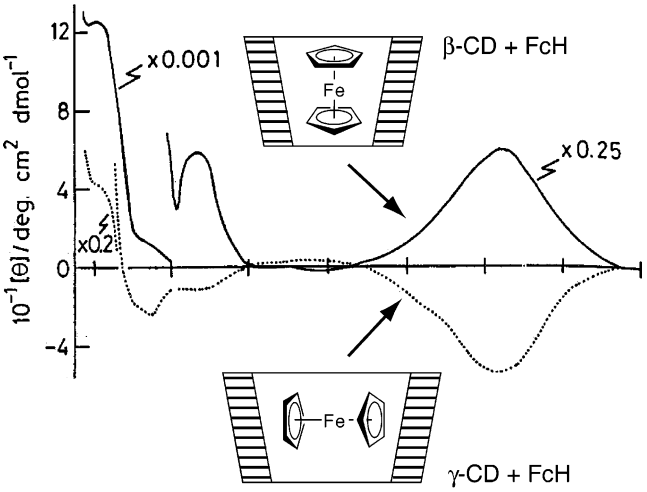


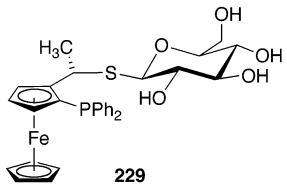
Figure 15. CD spectra of ferrocene complexed to β-CD and γ-CD as annotated. Reprinted with permission from ref 504. Copyright 1990 The Royal Society of Chemistry.

constants and thermodynamic parameters in various solvents, were also obtained by tethering the ferrocene moiety covalently to the cyclodextrin core.[505–507] Structural details were obtained from molecular modeling calculations by two groups.[508,509]

5.4. Other Carbohydrate Derivatives

Ferrocene derivatives have found widespread use as chiral auxiliaries with applications in asymmetric catalysis.[44,510] Albinati et al. reported the first use of a ferrocene-based chiral ligand **229** derived from thioglucose (Scheme 70).[511] A Pd(II) allyl complex of

Scheme 70. Ferrocene Sugar Chiral Auxiliary 229 for Pd-Catalyzed Allylic Alkylation



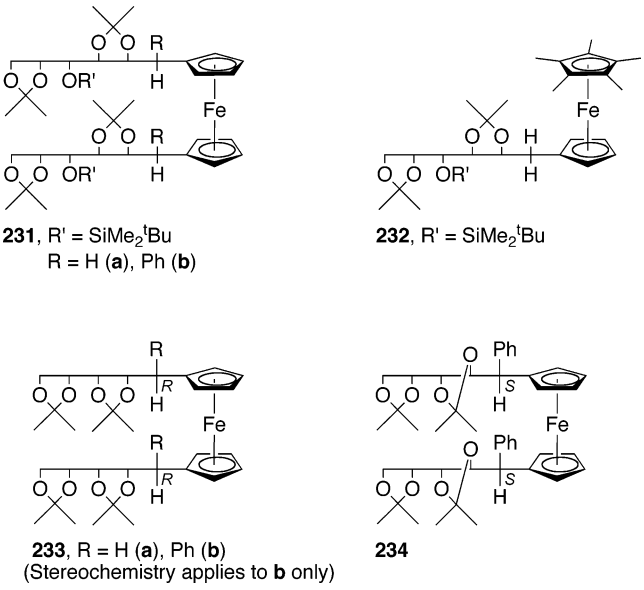
229

229 was used in the enantioselective allylic alkylation of PhCH=CHCH(OAc)Ph, giving a nonoptimized ee of 88%. The molecular basis for this enantioselectivity was readily explained by a crystal structure analysis of **229**.

So far, Vasella's group has prepared all of the chemically more unusual ferrocene sugar derivatives that we are aware of.[512–514] Cyclopentadienyl *C*-glycosides have been prepared as latent fulvenes.[512] $LiAlH_4$ reduction, followed by silyl ether formation, gave the fully protected cyclopentadienyl mannitols **230a**. In a similar fashion, reaction with PhLi, followed by silyl ether formation, gave **230b**. Lithiation of **230**, followed by addition of $FeCl_2$ or a Cp*Fe synthon, then gave the ferrocene derivatives **231a**,**b** and **232**. Separation of the three diastereomers of **231b**, which formed in a nonstatistical ratio, by preparative HPLC af-

forded these interesting metallocenes in stereochemically pure form (Scheme 71). The configura-

Scheme 71. C-Glycosidic Ferrocenylated Carbohydrates Prepared by Vasella and Co-workers



tion of these stereoisomers was deduced from ^{1}H NMR data. Related sequences of reactions gave the two metallocenes **233** and **234** derived fromribose and arabinose, respectively. It is of interest to note that stereochemically almost pure compounds **233b** were obtained in THF, in contrast to a low dia-stereomeric excess for **231b**. The stereochemistry at C-1 is stringently controlled in the phenylated pre-cursors of **233b** and evidently retained by the lithiation/metathesis reaction sequence. The resultof an X-ray structure analysis of **234** is shown in Figure 16.

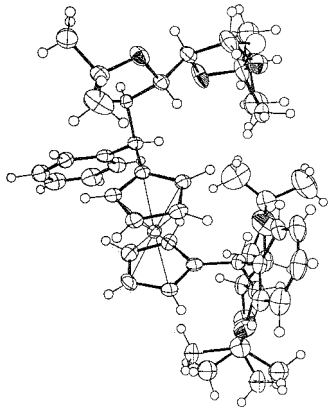
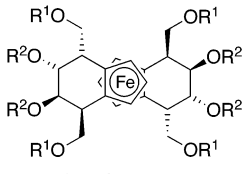


Figure 16. X-ray single-crystal structure of **234**. Reprinted with permission from ref 512. Copyright 1994 Wiley-VCH.

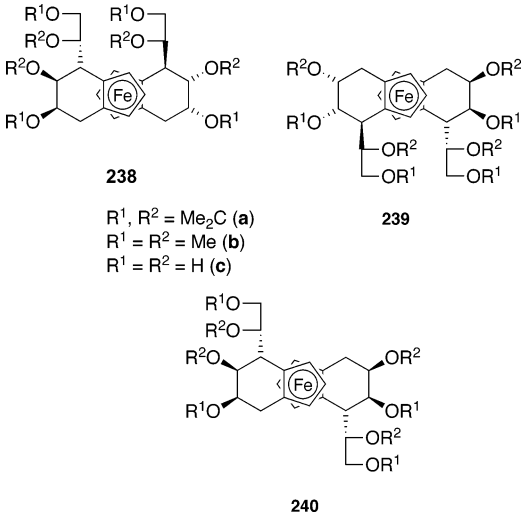
Annulated ferrocene derivatives of carbohydrates were reported by the same group.[513,514] The dimesylate of 1,3,4,6-tetra-*O*-methyl-D-mannitol **235** reacted with CpNa to give a spiro[4,4]nona-1,3-diene. Thermolysis of this compound afforded three isomeric tetrahydroindenes, which could be transformed into the annulated C_2-symmetric ferrocene **236a** by lithiation and reaction with $FeCl_2$.[514] In a related fashion, **236b** and **c** were obtained (Scheme 72).[513] C_1-Sym-

Scheme 72. Annulated Ferrocene Carbohydrate Derivatives 236



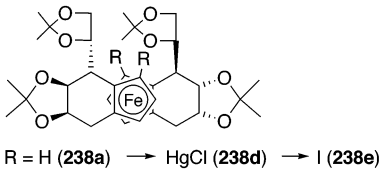
metric annulated ferrocenes were obtained from 2,3:5,6-di-*O*-isopropylidene-D-mannitol (**237**) or the corresponding tetra-*O*-methyl derivative.[513] In this case, a mixture of **238**, **239**, and **240** was obtained with a slight preference for **238a** and **239b**, respectively, over the statistical ratio (Scheme 73). The isopropyl-

Scheme 73. C_1-Symmetric Annulated Ferrocene Carbohydrate Derivatives



idene derivatives could be deprotected by HCl in MeOH, yielding **238c**−**240c**. All these deprotected derivatives were quite soluble in water (at least 100 g/L). **238a** was readily converted into the 1,1′-diiododerivative (**238e**) via chloromercuration (**238d**, Scheme 74). In this paper,[513] the crystal structures

Scheme 74. Derivatization of the Annulated Ferrocene Carbohydrate Derivative 238a



of **239a**, **240a**, and **238d** were reported along with thourough NMR characterization of all derivatives.

6. Ferrocene Conjugates with Other Biomolecules

In this chapter, ferrocene conjugates of biomolecules that differ from the classes in the previous sections are presented. Attention is only given to biomolecules that are important to mammals, such as hormones and molecules that are involved in the metabolism of lipids. We have undertaken no effort

Table 8. Overview of the Reported Ferrocene 17β-Estradiol Derivatives[a]

R_1	R_2	R_3	ref
O−CO−Fc	OH	H	515
OH	O−CO−Fc	H	515, 528
O−CO−Fc	O−CO−Fc	H	528
OH	O−CO−$(CH_2)_2$−CO−NH−CH_2−Fc	H	516, 517, 529
OH	O−CO−$(CH_2)_2$−CO−NH−CH_2−Fc	O−CO−$(CH_2)_2$−CO−NH−CH_2−Fc	517, 529
O−CH_2−CO−NH−CH_2−Fc	OH	H	529
O−CH_2−CO−NH−CH_2−Fc	OH	OH	529
OH	NH−CO−$(CH_2)_2$−CO−NH−CH_2−Fc	H	517
OCH_3	OH[a]	N=CH−Fc[b]	530[c]
OCH_3	OH[a]	NH−CH_2−Fc[b]	530[c]

[a] The 17α-ferrocenyl and 17α-ferrocenylethynyl derivatives **242** and **243** are not included in this table. [b] Also the compound with the inverted geometry at this carbon atom was reported. [c] X-ray crystal structure is reported in this reference.

to collect reports on the labeling of biomolecules that play an important role in lower organisms, such as plants (e.g. growth regulators) and insects (e.g pheromones).

6.1. Ferrocene Conjugates with Hormones

Peptide hormones such as enkephalin have already been treated in section 2.

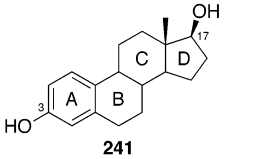
6.1.1. Steroid Hormones

The first reports of steroid hormones labeled with the ferrocene moiety occurred in the literature in 1977.[515,516] Riesselmann and Wenzel esterified ferrocene carboxylic acid (**40**) with the 3-OH group of estradiol and estrone.[515] Subsequently, metal exchange reactions with $^{103}RuCl_3$ afforded the radioactive ruthenocene estradiol and estrone conjugates. These derivatives were used to study the organ distribution and clearance properties in mice.

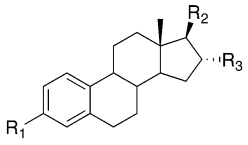
Cais and co-workers prepared ferrocene-labeled steroid derivatives for immunoassays.[516,517] For the first time, they showed that introduction of a metal complex on a biomolecule allows the quantification of unlabeled biomolecules in a competitive assay via atomic absorption spectroscopy. Their method is nowadays known under the term metallo immuno assay. Other metal-specific detection methods, such as IR spectroscopy (carbonyl metallo immuno assay, CMIA)[518,519] and amperometric detection (see below), have also been explored.

In particular, the steroid hormone estradiol **241** has been the subject of extensive labeling with the ferrocene group. An overview of the reported conjugates is given in Table 8 (see also Scheme 75). Two

Scheme 75. Estradiol 241 and Substituted Derivatives Thereof (bottom)



241



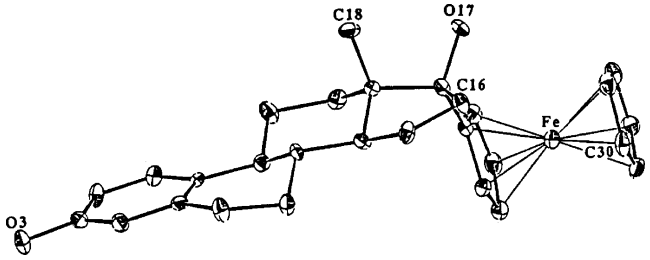
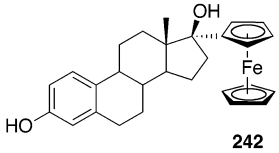


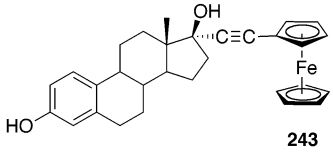
Figure 17. X-ray single-crystal structure of **242**. Reprinted (with modifications) with permission from ref 521. Copyright 1994 Elsevier.

very interesting ferrocene conjugates are not included in this table, namely 17α-ferrocenyl-17β-estradiol (**242**)[520−522] and 17α-ferrocenylethynyl-17β-estradiol (**243**);[523,524] see Scheme 76. The molecular structure

Scheme 76. Molecular Structure of 17α-Ferrocenyl-17β-estradiol (242) and 17α-Ferrocenylethynyl-17β-estradiol (243)



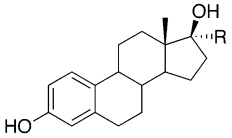
242



243

of **242** is depicted in Figure 17. Interestingly, these conjugates were found to have a relative binding affinity (RBA) of 8% and 28% to the estrogen α-receptor (ERα). These values are summarized in Table 9, together with several other mononuclear organometallic estradiol conjugates (Scheme 77). A chinese

Scheme 77. Estradiol Derivatives Tested for Receptor Binding (Table 9)



group has further introduced a ^3H radiolabel into **242**.[525]

Table 9. Relative Binding Affinity (RBA) of Some Mononuclear Organometallic Estradiol Derivatives to the ERα Receptor[a,b] (See Scheme 77)

R	RBA[a]	ref[b]
Fc	8	531[c]
(η-C_5H_4)Ru(Cp)	2	531[c]
-≡-Fc	28, 37[d]	524[c]
(η-C_6H_5)Cr(CO)$_3$	11	532
-≡-(η-C_6H_5)Cr(CO)$_3$	24	532
-≡-(η-C_5H_4)Mn(CO)$_3$	15	533
-≡-(η-C_5H_4)Re(CO)$_3$	16	533
CH_2-(η-C_5H_4)Mn(CO)$_3$	2.5	533
CH_2-(η-C_5H_4)Re(CO)$_3$	0.8	533
[(η-C_5H_4)Ru(Cp*)]OTf	0	532

[a] RBA values determined in a competitive radioreceptor binding assay at 0 °C. [b] For examples of estradiol conjugates with dinuclear organometallic complexes, see refs 534 and 535. [c] X-ray crystal structure reported. [d] RBA to the ERβ receptor.

The ERα is the first receptor subtype that was discovered and cloned.[526] In 1996, a second estrogen receptor, termed ERβ, was identified and cloned.[527] Thus far, only the affinity of 17α-ferrocenylethynyl-17β-estradiol for the ERβ has been determined to be 37% of that of estradiol (Table 9).

Two reports on a different female sexual hormone estrone **244** appeared in the literature (see Table 10

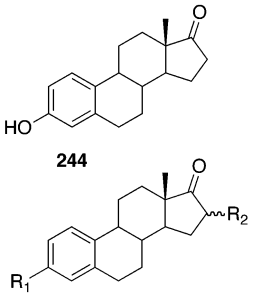
Table 10. Constitution of the Reported Estrone Derivatives (Scheme 78)

R_1	R_2	ref
OH	=CH−Fc	523
O−CH_2−CO−NH−CH_2−Fc	H	529

Table 11. Overview of the Reported Ferrocenylated Cholesterol Derivatives (Scheme 80)

R_1	R_2	ref
O−CO−Fc	H	528
O−CO−CH_2−Fc	H	538
O−CO−$(CH_2)_4$−Fc	H	538
NH−CH_2−Fc	H	530
N(Ac)−CH_2−Fc	H	530
NH−CH_2−Fc	OH	530

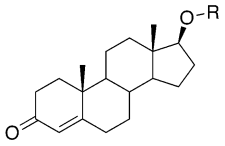
Scheme 78. Estrone 244 and Derivatives (botttom)



and Scheme 78).[523,529] In a different paper, Shimada et al. labeled the position 3 glucoronides of estradiol and estrogen with the Fem moiety for HPLC-ECD investigations.[536] In a related paper, the group of Shimada also investigated derivatization of hydroxysteroids, such as estrogen and dehydroepiandrosterone, with various ferrocene reagents.[537]

Only one example of a ferrocene-labeled conjugate **246** of the male sexual hormone testosterone **245** has been reported.[515,528] This derivative is shown in Scheme 79.

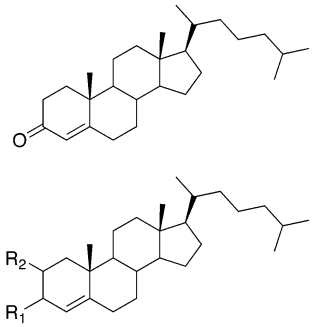
Scheme 79. Testosterone 245 and Derivatives (bottom)



R = H (testosterone, **245**)
R = CO-**Fc** (**246**)

A variety of ferrocene cholesterol derivatives have been prepared. An overview of these is given in Table 11 and Scheme 80. It should be noted that a steroid

Scheme 80. Cholesterol (top) and Derivatives (bottom, see Table 11)



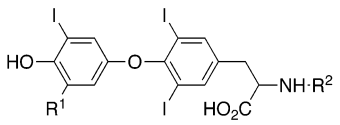
derivative with some resemblance to cholesterol has been reported quite recently by Coutoli-Argyropoulou et al. via Sonogashira coupling.[402]

In his Ph.D. work, Koller synthesized a variety of steroid derivatives in which a ferrocene nucleus served as the A-ring (see Scheme 75).[539] Several of these derivatives were screened for antibacterial and pepsin-inhibiting activity. At concentrations of 100 μg/mL, all of these compounds exhibited antibacterial properties, whereas only one was found to display significant pepsin-inhibiting activity.

6.1.2. Other Hormones

The two related thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) have been labeled with the ferrocene moiety (Scheme 81). Whereas the former

Scheme 81. Triiodothyronine and Thyroxin Conjugates



R^1 = H, R^2 = **Fc**-CO- (**247**)
R^1 = I, R^2 = **Fc**-CO- (**248**) or **Fc**-CH_2- (**249**)

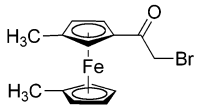
was only labeled with ferrocenecarboxylic acid (**40**) to yield **247**,[353] the latter was labeled with both the ferrocenoyl moiety, via amide formation with ferrocenecarboxylic acid (**248**),[540] and the Fem moiety, via imine formation with ferrocenecarbaldehyde (**67**) and subsequent reduction with $NaBH_4$ (**249**).[541] The T_3 and T_4 conjugates were employed in amperometric

immunoassays. The immunosensors constructed with the T_4 ferrocene derivatives allowed a detection limit for T_4 of 15 nM.[540,541]

6.2. Various Biomolecules

One class of biomolecules that has not been labeled extensively is molecules involved in the metabolism of lipids. For example, almost no conjugates of ferrocene with fatty acids have been reported thus far. Nambara and co-workers reported the determination of 10 fatty acids by HPLC-ECD after derivatization with 3-bromoacetyl-1,1′-dimethylferrocene (**250**) (Scheme 82).[542] The method showed a detection

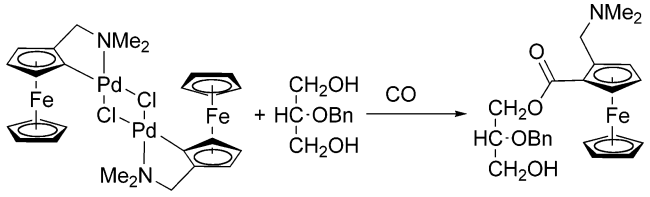
Scheme 82. A Ferrocene Reagent 250 That Was Used for the Derivatization of Fatty Acids



250

limit as low as 0.5 pmol and was suitable for direct determination of various fatty acids in human serum. A conjugate of ferrocene with another constituent of fats, namely glycerol, has been reported.[543,544] Sokolov and Troitskaya used the dimeric ferrocenyl-organopalladium complex **251** to synthesize optically active glycerol derivatives **252** via a diastereoselective carbonylation reaction (Scheme 83). The ferrocenoyl

Scheme 83. Synthesis of Glycerol Derivatives 252



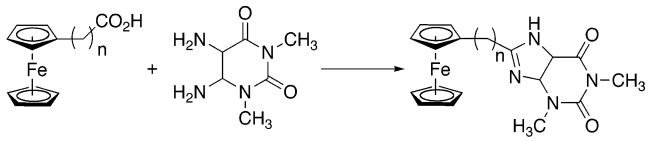
251 **252**

group functions in this case as a protecting group which can be removed by base hydrolysis.

An important molecule for the metabolism of phospholipids is choline. A ferrocenylcholine derivative has been prepared and tested for its ability to inhibit the hydrolysis of butyrylcholine by the enzyme horse serum butyrylcholinesterase.[545] The K_i of the ferrocenylcholine conjugate was determined to be 9.63×10^{-6} M/L.

In a very recent paper, the synthesis of ferrocene−theophylline conjugates for use in an electrochemical immunoassay is described.[546] Such a system was originally proposed using ferrocene−morphine conjugates[547] and has subsequently been applied to other drugs such as lidocaine.[548] Condensation of ferrocenylalkyl carboxylic acids with 5,6-diamino-1,3-dimethyluracil (**253**) yields ferrocenylalkyltheophylline conjugates **254** with different alkyl chain lengths (Scheme 84).[549] Functionalization of the unsubstituted Cp ring is also possible either by Friedel−Crafts acetylation or by methylation. A Mannich reaction yields tertiary amino derivatives, although as a mixture of isomers which could not be readily

Scheme 84. Ferrocenylalkyl Derivatives of Theophylline (254)



n = 1, 2, 3, 4, 8 **253** **254**

separated. The mixture was alkylated to yield more hydrophilic quarternary ferrocene ammonium salts with pendant theophylline side chains. The conjugates were evaluated in a homogeneous, competitive immunoassay for theophylline with amperometric detection.[546] The system was coupled to enzymatic glucose oxidase/glucose amplification. Unfortunately, the original system had poor sensitivity in the clinically relevant range of concentrations. The sensitivity could be improved, however, when the cationic Mannich conjugates of theophylline were used.

7. Future Perspectives

The work summarized in this article testifies impressively to the development of the field in the last 50 years. Chemical synthesis routes have been devised to create conjugates of ferrocene with all different kinds of biomolecules, as outlined in the various sections above. Naturally, much of this research was driven by chemical curiosity and the main objective was to "make" the compound or class of compounds. In addition, much effort has been devoted to structural and spectroscopic characterization.

For future work, we expect to see more functional characterization of ferrocene bioconjugates, along with synthetic efforts to arrive at one particular molecular target with projected properties. Such compounds should capitalize on the unique properties of the metallocene core. In terms of structural properties, this would certainly be the preorganization of the cyclopentadienyl rings at a fixed distance, along with a certain degree of freedom due to the unhindered rotation of the rings against each other. This might lead to a situation where a host is bound to a ferrocene conjugate in an "induced fit". Such a situation is analogous to certain proteins, where the conformation of the protein changes upon ligand binding and either a reaction or a molecular signal is initiated. The signal from ferrocene might, for instance, be a change in electrochemical properties.

The application of the very well behaved redox chemistry of ferrocene in electrochemical sensor devices is certainly another area that will blossom. Ferrocene-modified glucose oxidase has already been used in commercial instruments for the monitoring of glucose levels in patients with diabetes. We have indicated above the use of electrochemical DNA detection devices, some of which are, in principle, extendable to a chip-based array format. While several assay schemes have been explored, more work is needed until, hopefully, one day a marketable device emerges. However, this need not be limited to DNA/RNA detection alone. Many other applications are conceivable, for example in the detection of

carbohydrates, proteins, environmental pollutants, drugs, or other small molecules. We note that there is vast room for development in those areas.

In addition to sensors, the favorable electrochemical properties of ferrocene might be exploited for other applications as well. We could imagine electrochemically switchable ion channels, both as model systems for natural ion channels and as artificial nanomolecular devices that regulate transport. Electrochemically triggered reactions are another fascinating area where ferrocene bioconjugates might have an impact.

This review has not included medicinal applications of ferrocene. This is, of course, another area of active research. The key point is to use the specific and unique properties of ferrocene, rather than create a "benzene-like" derivative. In this area, a lot of biological testing has been performed already. Apart from a few scattered experiments that were indicated in the respective sections, this is not yet the case for the bioconjugates treated herein. On the other hand, it would be interesting to see more biological testing, for example on ferrocene peptide or ferrocene sugar conjugates. Unexpected and hopefully unusual activity may be expected in those areas. It would be highly gratifying to see a ferrocene-based drug or detection device on the market one day.

8. Acknowledgment

The authors are extremely grateful to Mrs. Karin Weiss for expert handling of over 500 references (or close to 30 kg of literature!) and providing all sorts of support during the preparation of this article. Mrs. J. Theurich (Max-Planck-Institut for Bioinorganic Chemistry, Mülheim) is acknowledged for ordering copies of a sizable portion of the literature cited in this article. N.M.-N. is grateful to all co-workers (present and past, long-term and short term) for their contribution to Bioorganometallic Chemistry and the success of our group. A number of colleagues have provided preprints and preliminary information, which is also gratefully acknowledged. The support of R. Alberto (University of Zurich) to D.R.v.S. in the final stages of this work is also appreciated.

9. List of Abbreviations

Standard three letter codes for amino acids are used throughout. Unless specifically noted, stereochemistry implies pure L-amino acids. Peptides are consistently written from N- to C-terminus in standard peptide nomenclature. In a peptide, "Gly" corresponds to the fragment "HN-CH_2-CO". For example, H-Gly-NH_2 is the carboxamide of glycine (H_2N-CH_2-$CONH_2$), Ac-Gly-Ala-OH is N-acetylated glycylalanine. According to common convention, the same four letter code is used for PNA as for DNA; small letters, however, indicate PNA oligomers.

A	adenine (nucleobase) or adenosine (nucleoside)
AA	amino acid
Ala	alanine
ALM	alamethicin
Asn	asparagine
AT	angiotensin
Bn	benzyl
BK	Bradykinin
Boc	*tert*-butoxycarbonyl
BR	bacteriorhodopsin
BSA	bovine serum albumin
C	cytosine (nucleobase) or cytidine (nucleoside)
CA	(1*R*,3*S*)-camphoric acid
CD	circular dichroism
CFPI	3-carboxy-4-ferrocenylphenylisothiocyanate
CGE	capillary gel electrophoresis
CHO cells	Chinese hamster ovarian cells
CMIA	carbonyl metallo immuno assay
Cp	cyclopentadienide anion ($C_5H_5^-$)
CPG	controlled pore glass
CV	cyclic voltammetry/cyclic voltammogram
CyP450	cytochrome P450
dA	deoxyadenosine (DNA/RNA monomer)
DAAO	D-amino acid oxidase
DCC	dicyclohexyl carbodiimide
ddUTP	dideoxyuridine triphosphate
DMAP	*N*,*N*′-dimethylaminopyridine
dsDNA	double stranded DNA
dT	deoxythymidine (DNA monomer)
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide
Enk	enkephalin
ER	estrogen receptor
ET	electron transfer
FAD	flavin adenine dinucleotide
Fc	ferrocenyl substituent ($CpFeC_5H_4$)
Fem	ferrocenylmethyl
Fer	ferrocenylalanine
Fmoc	fluorenylmethoxycarbonyl
G	guanine (nucleobase) or guanosine (nucleoside)
Gln	glutamine
Glu	glutamate
Gly	glycine
GOD	glucose oxidase
HBTU	*O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HOSu	*N*-hydroxysuccinimide
HPLC	high performance (pressure) liquid chromatography
HPLC-ECD	high performance (pressure) liquid chromatography with electrochemical detection
HRP	horseradish peroxidase
IBCF	isobutyl chloroformate
Ig	immunoglobulin
IgG	immunoglobulin G
LARIS	laser atomization resonance ionization spectroscopy
Leu	leucine
Lys	lysine
MEI	morpholino ethylisocyanide
ODN	oligo desoxy nucleotide (DNA oligomer)
Pac	phenoxy-acetyl
PEG	poly(ethylene glycol)
Phe	phenylalanine
pkDAAO	pig kidney D-amino acid oxidase
PNA	peptide nucleic acid
Pro	proline
PyAOP	7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate
QCM	quartz crystal microbalance

RBA	relative binding affinity
rgDAAO	D-amino acid oxidase from *Rhodoturula gracilis*
RIS	resonance ionization spectroscopy
Sar	sarcosine (*N*-methylglyine)
SDS	sodium *n*-dodecyl sulfate
SIRIS	sputter-initiated resonance ionization spectroscopy
SNP	single nucleotide polymorphism
SP	substance P
ssDNA	single stranded DNA
SV	sinusoidal voltammetry
SWV	square wave voltammogram
T	thymine (nucleobase, only in DNA and PNA, not RNA) or thymidine (nucleoside)
TBTU	*O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′*N*′-tetramethyluronium tetrafluoroborate
TCBoc	trichloro-*tert*-butoxycarbonyl
TFA	trifluoroacetic acid
T_m	melting temperature (measure of stability of oligonucleotide duplices)
Tyr	tyrosine
U	uracil (nucleobase, RNA only) or uridine (nucleoside)
UTP	uridine triphosphate
Val	valine
wt	wild-type
Z	benzyloxycarbonyl

10. References

(1) Kealy, T. J.; Pauson, P. L. *Nature* **1951**, *168*, 1039.
(2) Miller, S. A.; Tebboth, J. A.; Tremaine, J. F. *J. Chem. Soc.* **1952**, 632.
(3) Wilkinson, G.; Rosenblum, M.; Whiting, M. C.; Woodward, R. B. *J. Am. Chem. Soc.* **1952**, *74*, 2125.
(4) Fischer, E. O.; Pfab, W. *Z. Naturforsch.* **1952**, *7b*, 377.
(5) Woodward, R. B.; Rosenblum, M.; Whiting, M. C. *J. Am. Chem. Soc.* **1952**, *74*, 3458.
(6) Jaouen, G.; Vessières, A.; Butler, I. S. *Acc. Chem. Res.* **1993**, *26*, 361.
(7) Fish, R. H.; Jaouen, G. *Organometallics* **2003**, *22*, 2166.
(8) Metzler-Nolte, N. *Angew. Chem.* **2001**, *113*, 1072; *Angew. Chem., Int. Ed.* **2001**, *40*, 1040.
(9) Köpf-Maier, P.; Köpf, H. *Chem. Rev.* **1987**, *87*, 1137.
(10) Köpf-Maier, P.; Köpf, H. *Struct. Bond.* **1988**, *70*, 105.
(11) Shen, W.-C.; Beloussow, K.; Meirim, M. G.; Neuse, E. W.; Caldwell, G. *J. Inorg. Organomet. Polym.* **2000**, *10*, 51.
(12) Neuse, E. W. *Macromol. Symp.* **2001**, *172*, 127.
(13) Edwards, E. I.; Epton, R.; Marr, G. *J. Organomet. Chem.* **1976**, *122*, C49.
(14) Edwards, E. I.; Epton, R.; Marr, G. *J. Organomet. Chem.* **1976**, *107*, 351.
(15) Edwards, E. I.; Epton, R.; Marr, G. *Chem. Abstr.* **1977**, *87*, 728.
(16) Edwards, E. I.; Epton, R.; Marr, G.; Rogers, G. K.; Thompson, K. J. *Spec. Publ. Chem. Soc.* **1977**, *128*, 92.
(17) Edwards, E. I.; Epton, R.; Marr, G. *J. Organomet. Chem.* **1979**, *168*, 259.
(18) Simionescu, C.; Lixandru, T.; Tataru, L.; Mazilu, I.; Vata, M.; Luca, S. *J. Organomet. Chem.* **1983**, *252*, C43.
(19) Simionescu, C.; Lixandru, T.; Scutaru, D.; Vata, M. *J. Organomet. Chem.* **1985**, *292*, 269.
(20) Scutaru, D.; Mazilu, I.; Tataru, L.; Vata, M.; Lixandru, T. *J. Organomet. Chem.* **1990**, *406*, 183.
(21) Scutaru, D.; Tataru, L.; Mazilu, I.; Diaconu, E.; Lixandru, T.; Simionescu, C. *J. Organomet. Chem.* **1991**, *401*, 81.
(22) Scutaru, D.; Mazilu, I.; Vata, M.; Tataru, L.; Vlase, A.; Lixandru, T.; Simionescu, C. *J. Organomet. Chem.* **1991**, *401*, 87.
(23) Scutaru, D.; Tataru, L.; Mazilu, I.; Vata, M.; Lixandru, T.; Simionescu, C. *Appl. Organomet. Chem.* **1993**, *7*, 225.
(24) Epton, R.; Marr, G.; Rogers, G. K. *J. Organomet. Chem.* **1976**, *110*, C42.
(25) Epton, R.; Marr, G.; Rogers, G. K. *J. Organomet. Chem.* **1978**, *150*, 93.
(26) Sawamura, M.; Sasaki, H.; Nakata, T.; Ito, Y. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2725.
(27) Biot, C.; Glorian, G.; Maciejewski, L. A.; Brocard, J. S.; Domarle, O.; Blampain, G.; Millet, P.; Georges, A. J.; Abessolo, H.; Dive, D.; Lebibi, J. *J. Med. Chem.* **1997**, *40*, 3715.
(28) Biot, C.; Caron, S.; Maciejewski, L. A.; Brocard, J. S. *J. Labelled Compd. Radiopharm.* **1998**, *51*, 911.
(29) Domarle, O.; Blampain, G.; Agnaniet, H.; Nzadiyabi, T.; Lebibi, J.; Brocard, J. S.; Maciejewski, L. A.; Biot, C.; Georges, A. J.; Millet, P. *Antimicrob. Agents Chemother.* **1998**, *42*, 540.
(30) Biot, C.; Delhaes, L.; Abessolo, H.; Domarle, O.; Maciejewski, L. A.; Mortuaire, M.; Delcourt, P.; Deloron, P.; Camus, D.; Dive, D.; Brocard, J. S. *J. Organomet. Chem.* **1999**, *589*, 59.
(31) Biot, C.; Delhaes, L.; N'Diaye, C. M.; Maciejewski, L. A.; Camus, D.; Dive, D.; Brocard, J. S. *Bioorg. Med. Chem.* **1999**, *7*, 2843.
(32) Delhaes, L.; Abessolo, H.; Biot, C.; Berry, L.; Delcourt, P.; Maciejewski, L. A.; Brocard, J. S.; Camus, D.; Dive, D. *Parasitol. Res.* **2001**, *87*, 239.
(33) Biot, C.; Delhaes, L.; Maciejewski, L. A.; Mortuaire, M.; Camus, D.; Dive, D.; Brocard, J. S. *Eur. J. Med. Chem.* **2000**, *35*, 707.
(34) Delhaes, L.; Biot, C.; Berry, L.; Maciejewski, L. A.; Camus, D.; Brocard, J. S.; Dive, D. *Bioorg. Med. Chem.* **2000**, *8*, 2739.
(35) Pradines, B.; Fusai, T.; Daries, W.; Laloge, V.; Rogier, C.; Millet, P.; Panconi, E.; Kombila, M.; Parzy, D. *J. Antimicrob. Chemother.* **2001**, *48*, 179.
(36) Pradines, B.; Tall, A.; Rogier, C.; Spiegel, A.; Mosnier, J.; Marrama, L.; Fusai, T.; Millet, P.; Panconi, E.; Trape, J. F.; Parzy, D. *Trop. Med. Int. Health* **2002**, *7*, 265.
(37) Delhaes, L.; Biot, C.; Berry, L.; Delcourt, P.; Maciejewski, L. A.; Camus, D.; Brocard, J. S.; Dive, D. *ChemBioChem* **2002**, *3*, 418.
(38) Atteke, C.; Ndong, J. M. M.; Aubouy, A.; Maciejewski, L. A.; Brocard, J. S.; Lebibi, J.; Deloron, P. *J. Antimicrob. Chemother.* **2003**, *51*, 1021.
(39) Beagley, P.; Blackie, M. A. L.; Chibale, K.; Clarkson, C.; Meijboom, R.; Moss, J. R.; Smith, P. J.; Su, H. *Dalton Trans.* **2003**, 3046.
(40) Jaouen, G.; Top, S.; Vessières, A.; Leclercq, G.; Quivy, J.; Jin, L.; Croisy, A. *C. R. Acad. Sci. Paris* **2000**, *Série IIc*, 89.
(41) Top, S.; Vessières, A.; Cabestaing, C.; Laios, I.; Leclercq, G.; Provot, C.; Jaouen, G. *J. Organomet. Chem.* **2001**, *637*−*639*, 500.
(42) Top, S.; Vessières, A.; Leclercq, G.; Quivy, J.; Tang, J.; Vaissermann, J.; Huché, M.; Jaouen, G. *Chem. Eur. J.* **2003**, *9*, 5223.
(43) Schlögl, K. *Monatsh. Chem.* **1957**, *88*, 601.
(44) Togni, A.; Hayashi, T. *Ferrocenes: Homogeneous Catalysis, Organic Synthesis, Material Science*; VCH: Weinheim, 1995.
(45) Severin, K.; Bergs, R.; Beck, W. *Angew. Chem.* **1998**, *110*, 1722; *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 1634.
(46) Ryabov, A. D. *Angew. Chem.* **1991**, *103*, 945; *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 931−941.
(47) Salmain, M.; Jaouen, G. *C. R. Chim.* **2003**, *6*, 249.
(48) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1972**, *58*, 323.
(49) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1974**, *79*, 223.
(50) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1976**, *106*, 259.
(51) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1976**, *123*, 205.
(52) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1978**, *147*, 273.
(53) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1979**, *167*, 53.
(54) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1980**, *189*, 163.
(55) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1981**, *211*, 215.
(56) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1982**, *227*, 373.
(57) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1982**, *237*, 161.
(58) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1983**, *257*, 209.
(59) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1984**, *278*, 255.
(60) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1986**, *298*, 133.
(61) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1987**, *318*, 231.
(62) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1988**, *343*, 79.
(63) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1988**, *357*, 247.
(64) Marr, G.; Rockett, B. W. *J. Organomet. Chem.* **1990**, *392*, 93.
(65) Rockett, B. W.; Marr, G. *J. Organomet. Chem.* **1991**, *416*, 327.
(66) Kochetkova, N. S.; Krynkina, Y. K. *Russ. Chem. Rev.* **1978**, *47*, 486; Translation from *Usp. Khim.* **1978**, *47*, 934.
(67) Dombrowski, K. E.; Baldwin, W.; Sheats, J. E. *J. Organomet. Chem.* **1986**, *302*, 281.
(68) Wang, J. *Electroanalysis* **2001**, *13*, 983.
(69) Willner, I.; Willner, B. *Bioelectrochem. Bioenerg.* **1997**, *42*, 43.
(70) Willner, I.; Katz, E. *Angew. Chem., Int. Ed.* **2000**, *39*, 1180; *Angew. Chem.* **2000**, *112*, 1230.
(71) Zatsepin, T. S.; Andreev, S. Y.; Hianik, T.; Oretskaya, T. S. *Russ. Chem. Rev.* **2003**, *72*, 537; Translation from *Usp. Khim*.
(72) Hauser, C. R.; Lindsay, J. K. *J. Org. Chem.* **1957**, *22*, 1246.
(73) Osgerby, J. M.; Pauson, P. L. *J. Chem. Soc.* **1958**, 656.
(74) Jackson, R. F. W.; Turner, D.; Block, M. H. *Synlett* **1996**, 862.
(75) Brunner, H.; König, W.; Nuber, B. *Tetrahedron: Asymmetry* **1993**, *4*, 699.
(76) Pospisek, J.; Toma, S.; Fric, I.; Blaha, K. *Collect. Czech. Chem. Commun.* **1980**, *45*, 435.
(77) Kira, M.; Matsubara, T.; Shinohara, H.; Sisido, M. *Chem. Lett.* **1997**, 89.
(78) Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. *Nature* **1975**, *258*, 577.
(79) Clement-Jones, V.; Besser, G. M. In *The Peptides: Analysis, Synthesis, Biology*; Udenfried, S., Meienhofer, J., Eds.; Academic Press: New York, 1984; Vol. 6, p 323.
(80) Cuignet, E.; Sergheraert, C.; Tartar, A.; Dautrevaux, M. *J. Organomet. Chem.* **1980**, *195*, 325.
(81) Epton, R.; Marr, G.; Willmore, G. A.; Hudson, D.; Snell, P. H.; Snell, C. R. *Int. J. Biol. Macromol.* **1981**, *3*, 395.

(82) Cuignet, E.; Dautrevaux, M.; Sergheraert, C.; Tartar, A.; Attali, B.; Cros, J. *Eur. J. Med. Chem.* **1982**, *17*, 203.
(83) Morley, J. S. *Annu. Rev. Pharmacol. Toxicol.* **1980**, *20*, 81.
(84) von Euler, U. S.; Gaddum, J. H. *J. Physiol.* **1931**, *72*, 74.
(85) Chang, M. M.; Leeman, S. E.; Niall, H. D. *Nature New Biol.* **1971**, *232*, 86.
(86) Tregear, G. W.; Niall, H. D.; Potts Jun, J. T.; Leeman, S. E.; Chang, M. M. *Nature New Biol.* **1971**, *232*, 87.
(87) Otsuka, M.; Yoshioka, K. *Physiol. Rev.* **1993**, *73*, 229.
(88) Brunet, J. C.; Cuignet, E.; Dautrevaux, M.; Demarly, A.; Gras, H.; Marcincal, P.; Sergheraert, C.; Tartar, A.; Vanvoorde, J. C.; Vanpoucke, M. In *Peptides 1980. Proceedings of the sixteenth European Peptide Symposium*; Brunfeldt, K., Ed.; Scriptor: Copenhagen, 1981; p 603.
(89) Rhaleb, N.-E.; Drapeau, G.; Dion, S.; Jukic, D.; Rouissi, N.; Regoli, D. *Br. J. Pharmacol.* **1990**, *99*, 445.
(90) Taylor, J. E.; DeFeudis, F. V.; Moreau, J. P. *Drug Dev. Res.* **1989**, *16*, 1.
(91) Couture, R.; Drouin, J.-N.; Leukart, O.; Regoli, D. *Can. J. Physiol. Pharmacol.* **1979**, *57*, 1437.
(92) Couture, R.; Fournier, A.; Magnan, J.; St-Pierre, S.; Regoli, D. *Can. J. Physiol. Pharmacol.* **1979**, *57*, 1427.
(93) Saavedra, J. M. *Endocrine Rev.* **1992**, *13*, 329.
(94) Maes, P.; Ricouart, A.; Escher, E.; Tartar, A.; Sergheraert, C. *Collect. Czech. Chem. Commun.* **1988**, *53*, 2914.
(95) Tartar, A.; Demarly, A.; Sergheraert, C.; Escher, E. In *Proceedings of the 8th American Peptide Symposium*; Hruby, V. J., Rich, D. H., Eds.; Pierce Chemical: Rockford, IL, 1983; p 377.
(96) Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145.
(97) Steitz, T. A.; Henderson, R.; Blow, D. M. *J. Mol. Biol.* **1969**, *46*, 337.
(98) Hanzlik, R. P.; Soine, P.; Soine, W. H. *J. Med. Chem.* **1979**, *22*, 424.
(99) Soine, W. H. *Diss. Abstr. Int.* **1979**, *39*, 3849.
(100) Kaluz, S.; Toma, S. *Collect. Czech. Chem. Commun.* **1988**, *53*, 638.
(101) Carlström, A.-S.; Frejd, T. *J. Org. Chem.* **1990**, *55*, 4175.
(102) Basu, B.; Chattopadhyay, S. K.; Ritzen, A.; Frejd, T. *Tetrahedron: Asymmetry* **1997**, *8*, 1841.
(103) Maricic, S.; Ritzen, A.; Berg, U.; Frejd, T. *Tetrahedron* **2001**, *57*, 6523.
(104) Maricic, S.; Berg, U.; Frejd, T. *Tetrahedron* **2002**, *58*, 3085.
(105) Carlström, A.-S.; Frejd, T. *Synthesis* **1989**, 414.
(106) Maricic, S.; Frejd, T. *J. Org. Chem.* **2002**, *67*, 7600.
(107) Smith, G. D.; Griffin, J. F. *Science* **1978**, *199*, 1214.
(108) Picone, D.; D'Ursi, A.; Motta, A.; Tancredi, T.; Temussi, P. A. *Eur. J. Biochem.* **1990**, *192*, 433.
(109) Adamczyk, M.; Reddy, R. E.; Rege, S. D. *Synth. Commun.* **2000**, *30*, 1389.
(110) Dialer, H.; Steglich, W.; Beck, W. *Tetrahedron* **2001**, *57*, 4855.
(111) Dialer, H.; Polborn, K.; Ponikwar, W.; Sünkel, K.; Beck, W. *Chem. Eur. J.* **2002**, *8*, 691.
(112) Kunz, D.; Fröhlich, R.; Erker, G. *Organometallics* **2001**, *20*, 572.
(113) Butler, I. R.; Quayle, S. C. *J. Organomet. Chem.* **1998**, *552*, 63.
(114) Okamura, T.; Sakauye, K.; Ueyama, N.; Nakamura, A. *Inorg. Chem.* **1998**, *37*, 6731.
(115) Heinze, K.; Schlenker, M. *Eur. J. Inorg. Chem.* **2004**, 2974.
(116) Barisic, L.; Rapić, V.; Kovac, V. *Croat. Chem. Acta* **2002**, *75*, 199.
(117) Pavlovic, G.; Barisic, L.; Rapić, V.; Leban, I. *Acta Crystallogr.* **2002**, *E58*, m13.
(118) Pavlovic, G.; Barisic, L.; Rapić, V.; Kovac, V. *Acta Crystallogr.* **2003**, *C59*, m55.
(119) Barisic, L.; Dropucic, M.; Rapić, V.; Pritzkow, H.; Kirin, S.; Metzler-Nolte, N. *J. Chem. Soc., Chem. Commun.* **2004**, 2004.
(120) Barisic, L.; Rapić, V.; Pritzkow, H.; Pavlovic, G.; Nemet, I. *J. Organomet. Chem.* **2003**, *682*, 131.
(121) Herrick, R. S.; Jarret, R. M.; Curran, T. P.; Dragoli, D. R.; Flaherty, M. B.; Lindyberg, S. E.; Slate, R. A.; Thornton, L. C. *Tetrahedron Lett.* **1996**, *37*, 5289.
(122) Kraatz, H.-B.; Lusztyk, J.; Enright, G. D. *Inorg. Chem.* **1997**, *36*, 2400.
(123) van Staveren, D. R.; Weyhermüller, T.; Metzler-Nolte, N. *Dalton Trans.* **2003**, 210.
(124) van Staveren, D. R. Ph.D. Thesis, Ruhr-Universität, Bochum, Germany, 2001.
(125) Bauer, W.; Polborn, K.; Beck, W. *J. Organomet. Chem.* **1999**, *579*, 269.
(126) Nomoto, A.; Moriuchi, T.; Yamazaki, S.; Ogawa, A.; Hirao, T. *J. Chem. Soc., Chem. Commun.* **1998**, 1963.
(127) Moriuchi, T.; Nomoto, A.; Yoshida, K.; Hirao, T. *J. Organomet. Chem.* **1999**, *589*, 50.
(128) Saweczko, P.; Enright, G. D.; Kraatz, H.-B. *Inorg. Chem.* **2001**, *40*, 4409.
(129) Kraatz, H.-B.; Leek, D. M.; Houmama, A.; Enright, G. D.; Lusztyk, J.; Wayner, D. D. M. *J. Organomet. Chem.* **1999**, *589*, 38.
(130) Cowan, P. M.; McGavin, S. *Nature* **1955**, *176*, 501.
(131) Steinberg, I. Z.; Harrington, W. F.; Berger, A.; Sela, M.; Katchalski, E. *J. Am. Chem. Soc.* **1960**, *82*, 5263.
(132) Galka, M. M.; Kraatz, H.-B. *ChemPhysChem* **2002**, 356.
(133) Bediako-Amoa, I.; Silerova, R.; Kraatz, H.-B. *J. Chem. Soc., Chem. Commun.* **2002**, 2430.
(134) Saweczko, P.; Kraatz, H.-B. *Coord. Chem. Rev.* **1999**, *190*−*192*, 185.
(135) Kraatz, H.-B.; Galka, M. *Metal Ions Biol. Syst.* **2001**, *38*, 385.
(136) Baker, M. V.; Kraatz, H.-B.; Quail, J. W. *New J. Chem.* **2001**, *25*, 427.
(137) Roberts, R. M. G.; Silver, J. *J. Organomet. Chem.* **1984**, *263*, 235.
(138) Greenwood, N. N.; Gibb, T. C. *Mössbauer Spectroscopy*; Chapman and Hall: London, 1971.
(139) Hess, A.; Metzler-Nolte, N. *J. Chem. Soc., Chem. Commun.* **1999**, 885.
(140) Hublau, P.; Sergheraert, C.; Ballester, L.; Dautrevaux, M. *Eur. J. Med. Chem.* **1983**, *18*, 131.
(141) Wieckowska, A.; Bilewicz, R.; Misicka, A.; Pietraszkiewicz, M.; Bajdor, K.; Piela, L. *Chem. Phys. Lett.* **2001**, *350*, 447.
(142) Pietraszkiewicz, M.; Wieckowska, A.; Bilewicz, R.; Misicka, A.; Piela, L.; Bajdor, K. *Mater. Sci. Eng. C* **2001**, *18*, 121.
(143) Iijima, S.; Mizutani, F.; Yabuki, S.; Tanaka, Y.; Asai, M.; Katsura, T.; Hosaka, S.; Ibonai, M. *Anal. Chim. Acta* **1993**, *281*, 483.
(144) Gallagher, J. F.; Kenny, P. T. M.; Sheehy, M. J. *Inorg. Chem. Commun.* **1999**, *2*, 200.
(145) Gallagher, J. F.; Kenny, P. T. M.; Sheehy, M. J. *Acta Crystallogr.* **1999**, *C55*, 1257.
(146) Eckert, H.; Koller, M. *Z. Naturforsch.* **1990**, *45b*, 1709.
(147) Wenzel, M.; Park, I.-H. *Appl. Radiat. Isot.* **1986**, *37*, 491.
(148) Cox, R. L.; Schneider, T. W.; Koppang, M. D. *Anal. Chim. Acta* **1992**, *262*, 145.
(149) Gallagher, J. F.; Kenny, P. T. M.; Sheehy, M. J. *Inorg. Chem. Commun.* **1999**, *2*, 327.
(150) Oberhoff, M.; Duda, L.; Karl, J.; Mohr, R.; Erker, G.; Fröhlich, R.; Grehl, M. *Organometallics* **1996**, *15*, 4005.
(151) Mandal, H. S.; Kraatz, H.-B. *J. Organomet. Chem.* **2003**, *674*, 32.
(152) Kraatz, H.-B. *Macromol. Symp.* **2003**, *196*, 39.
(153) Moriuchi, T.; Nomoto, A.; Yoshida, K.; Hirao, T. *Organometallics* **2001**, *20*, 1008.
(154) Moriuchi, T.; Nomoto, A.; Yoshida, K.; Ogawa, A.; Hirao, T. *J. Am. Chem. Soc.* **2001**, *123*, 68.
(155) Moriuchi, T.; Yoshida, K.; Hirao, T. *J. Organomet. Chem.* **2001**, *637*−*639*, 75.
(156) Plumb, K.; Kraatz, H.-B. *Bioconjugate Chem.* **2003**, *14*, 601.
(157) Sek, S.; Moszynski, R.; Seipiol, A.; Misicka, A.; Bilewicz, R. *J. Electroanal. Chem.* **2003**, *550*−*551*, 359.
(158) Georgopoulou, A. S.; Mingos, D. M. P.; White, A. J. P.; Williams, D. J.; Horrocks, B. R.; Houlton, A. *J. Chem. Soc., Dalton Trans.* **2000**, 2969.
(159) Corain, B.; Longato, B.; Favero, G.; Ajó, D.; Pilloni, G.; Russo, U.; Kreissel, F. R. *Inorg. Chim. Acta* **1989**, *157*, 259.
(160) Moriuchi, T.; Yoshida, K.; Hirao, T. *Organometallics* **2001**, *20*, 3101.
(161) Moriuchi, T.; Yoshida, K.; Hirao, T. *J. Organomet. Chem.* **2003**, *668*, 31.
(162) Moriuchi, T.; Hirao, T. *J. Synth. Org. Chem. Jpn.* **2001**, *59*, 1195.
(163) Dialer, H.; Steglich, W.; Beck, W. *Z. Naturforsch.* **2001**, *56B*, 1084.
(164) Han, Q.-W.; Zhu, X.-Q.; Hu, X.-B.; Cheng, J.-P. *Chem. J. Chin. Univ.* **2002**, *23*, 2076.
(165) Huang, H.; Mu, L.; He, J.; Cheng, J.-P. *J. Org. Chem.* **2003**, *68*, 7605.
(166) Chowdhury, S.; Schatte, G.; Kraatz, H.-B. *J. Chem. Soc., Dalton Trans.* **2004**, 1726.
(167) Bediako-Amoa, I.; Sutherland, T. C.; Li, C.-Z.; Silerova, R.; Kraatz, H.-B. *J. Phys. Chem.* **2004**, *B108*, 704.
(168) Xu, Y.; Saweczko, P.; Kraatz, H.-B. *J. Organomet. Chem.* **2001**, *637*−*639*, 335.
(169) Xu, Y.; Kraatz, H.-B. *Tetrahedron Lett.* **2001**, *42*, 2601.
(170) Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359.
(171) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. *J. Antibiot.* **1970**, *23*, 259.
(172) Katoh, I.; Yasunaga, T.; Ikawa, Y.; Yoshinaka, Y. *Nature* **1987**, *329*, 654.
(173) Schachschneider, G.; Wenzel, M. *J. Labelled Compd. Radiopharm.* **1984**, *22*, 235.
(174) Kitagawa, K.; Morita, T.; Kawasaki, M.; Kimura, S. *J. Polym. Sci. A* **2003**, *41*, 3493−3500.
(175) Kraatz, H.-B. Personal communication.
(176) Osman, A. M.; El-Maghraby, M. A.; Hassan, K. M. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 2226.
(177) Freiesleben, D.; Polborn, K.; Robl, C.; Sünkel, K.; Beck, W. *Can. J. Chem.* **1995**, *73*, 1164.
(178) Hess, A.; Sehnert, J.; Weyhermüller, T.; Metzler-Nolte, N. *Inorg. Chem.* **2000**, *39*, 5437.
(179) Khruscheva, N. S.; Loim, N. M.; Sokolov, V. I. *Russ. Chem. Bull.* **1999**, *48*, 578; Translation from *Izv. Akad. Nauk Ser. Khim.* **1999**, 583.

(180) Kayser, B.; Altman, J.; Nöth, H.; Knizek, J.; Beck, W. *Eur. J. Inorg. Chem.* **1998**, 1791.
(181) Eckert, H.; Forster, B.; Seidel, C. *Z. Naturforsch.* **1991**, *46b*, 339.
(182) Eckert, H.; Seidel, C. *Angew. Chem.* **1986**, *98*, 168; *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 159.
(183) Sehnert, J.; Hess, A.; Metzler-Nolte, N. *J. Organomet. Chem.* **2001**, *637*−*639*, 349.
(184) Hess, A. Ph.D. Thesis, Ruhr-Universität, Bochum, Germany, 1999.
(185) Hess, A.; Brosch, O.; Weyhermüller, T.; Metzler-Nolte, N. *J. Organomet. Chem.* **1999**, *589*, 75.
(186) Sonogashira, K.; Tohda, Y.; Hagihara, N. *Tetrahedron Lett.* **1975**, *50*, 4467.
(187) Hagihara, N.; Sonogashira, K.; Tohda, Y. *Synthesis* **1977**, 777.
(188) Hagihara, N.; Takahashi, S.; Kuroyama, Y.; Sonogashira, K. *Synthesis* **1980**, 627.
(189) Brosch, O.; Weyhermüller, T.; Metzler-Nolte, N. *Inorg. Chem.* **1999**, *38*, 5308.
(190) Brosch, O.; Weyhermüller, T.; Metzler-Nolte, N. *Eur. J. Inorg. Chem.* **2000**, 323.
(191) Brosch, O. Ph.D. Thesis, Ruhr-Universität, Bochum, Germany, 1999.
(192) Meienhofer, J. *The Mixed Carbonic Anhydride Method of Peptide Synthesis*; Academic Press: London, 1979.
(193) Schmitt, J. D.; Sansom, M. S. P.; Kerr, I. D.; Lunt, G. G.; Eisenthal, R. *Biochemistry* **1997**, *36*, 1115.
(194) Fox, R. O., Jr.; Richards, F. M. *Nature* **1982**, *300*, 325.
(195) Latorre, R.; Alvarez, O. *Physiol. Rev.* **1981**, *61*, 77.
(196) Woolley, G. A.; Wallace, B. A. *Biochemistry* **1993**, *32*, 9819.
(197) Sansom, M. S. P. *Q. Rev. Biophys.* **1993**, *26*, 365.
(198) Bianco, A.; Gasparrini, F.; Maggini, M.; Misiti, D.; Polese, A.; Prato, M.; Scorrano, G.; Toniolo, C.; Villani, C. *J. Am. Chem. Soc.* **1997**, *119*, 7550.
(199) Guldi, D. M.; Maggini, M.; Scorrano, G.; Prato, M.; Bianco, A.; Toniolo, C. *J. Inf. Rec.* **1998**, *24*, 33.
(200) Bianco, A.; Corvaja, C.; Crisma, M.; Guldi, D. M.; Maggini, M.; Sartori, E.; Toniolo, C. *Chem. Eur. J.* **2002**, *8*, 1544.
(201) Malisza, K. L.; Top, S.; Vaissermann, J.; Caro, B.; Senechal-Tocquer, M.-C.; Senechal, D.; Saillard, J.-Y.; Triki, S.; Kahlal, S.; Britten, J. F.; McGlinchey, M. J.; Jaouen, G. *Organometallics* **1995**, *14*, 5273.
(202) Savage, D.; Gallagher, J. F.; Ida, Y.; Kenny, P. T. M. *Inorg. Chem. Commun.* **2002**, *5*, 1034.
(203) Eckert, H.; Koller, M. *J. Liq. Chromatogr.* **1990**, *13*, 3399.
(204) German Patent 39193178-A1, 1990; *Chem. Abstr.* **1991**, *115*, 25536.
(205) White, M. W. *J. Chromatogr.* **1983**, *262*, 420.
(206) Narasimhachari, N. *J. Liq. Chromatogr.* **1986**, *9*, 2223.
(207) Iriyama, K.; Yoshiura, M.; Iwamoto, T. *J. Liq. Chromatogr.* **1986**, *9*, 2955.
(208) Drumheller, A. L.; Bachelard, H.; St-Pierre, S.; Jolicoeur, F. B. *J. Liq. Chromatogr.* **1985**, *8*, 1829.
(209) Iriyama, K.; Iwamoto, T.; Yoshiura, M. *J. Liq. Chromatogr.* **1986**, *9*, 955.
(210) Fleming, L. H.; Reynolds, N. C., Jr. *J. Chromatogr.* **1988**, *431*, 65.
(211) Shimada, K.; Kawai, Y.; Oe, T.; Nambara, T. *J. Liq. Chromatogr.* **1989**, *12*, 359.
(212) Di Gleria, K.; Hill, H. A. O.; Wong, L.-L. *FEBS Lett.* **1996**, *390*, 142.
(213) Katayama, Y.; Sakakihara, S.; Maeda, M. *Anal. Sci.* **2001**, *17*, 17.
(214) Lo, K. K.-W.; Lau, J. S.-Y.; Ng, D. C.-M.; Zhu, N. *Dalton Trans.* **2002**, 1753.
(215) Duy, T. N.; Lam, P. K. S.; Shaw, G. R.; Connell, D. W. *Rev. Environ. Contam. Toxicol.* **2000**, *163*, 113.
(216) Lo, K. K.-W.; Ng, D. C.-M.; Lau, J. S.-Y.; Wu, R. S.-S.; Lam, P. K.-S. *New J. Chem.* **2003**, *27*, 274.
(217) Eckert, H.; Kiesel, Y.; Seidel, C.; Kaulberg, C.; Brinkmann, H. In *Chemistry of Peptides and Proteins*; Voelter, W., Bayer, E., Ovchinnikov, Y. A., Ivanov, T., Eds.; Walter de Gruyter: Berlin, New York, 1986; Vol. 3, p 19.
(218) Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: Berlin, 1984.
(219) Jones, J. *The Chemical Synthesis of Peptides*; Clarendon Press: Oxford, U.K., 1991.
(220) Stewart, A. S. J.; Drey, C. N. C. *J. Chem. Soc., Perkin Trans. 1* **1990**, 1753.
(221) Drey, C. N. C.; Stewart, A. S. J. In *Peptides 1986*; Theodoropoulos, D., Ed.; Walter de Gruyter: Berlin, 1987; p 65.
(222) Ugi, I. *Angew. Chem.* **1959**, *71*, 386.
(223) Ugi, I. *Angew. Chem.* **1962**, *74*, 9.
(224) Sjoberg, K. *Sven. Kem. Tidskr.* **1963**, *75*, 493; *Chem. Abstr.* **1964**, *60*, 5290b.
(225) Gambaryan, N. P. *Zh. Vses. Khim. Ova. im. D. I. Mendeleeva* **1967**, *12*, 65; *Chem. Abstr.* **1967**, *66*, 94400t.
(226) Hoffmann, P.; Marquarding, D.; Kleimann, H.; Ugi, I. In *The chemistry of the cyano group*; Rappoport, Z., Ed.; Interscience Publishers, a division of John Wiley & Sons: London, 1970; p 853.
(227) Gokel, G.; Lüdke, G.; Ugi, I. In *Isonitrile Chemistry*; Ugi, I., Ed.; Academic Press: New York, 1971; p 145.
(228) Ugi, I. In *Neuere Methoden der präparativen organischen Chemie*; Foerst, W., Ed.; Verlag Chemie: Weinheim, 1966; p 1.
(229) Bodanszky, M. *Principles of Peptide Synthesis*; Springer-Verlag: Berlin, 1984.
(230) Ugi, I. In *The Peptides—Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. 2—Special methods in Peptide Synthesis, Part A, p 365.
(231) Ugi, I.; Marquarding, D.; Urban, R. In *Chemistry and Biochemistry of Amino acids, Peptides and Proteins*; Weinstein, B., Ed.; Marcel Dekker: New York, 1982; Vol. 6, p 245.
(232) Ugi, I. *Rec. Chem. Prog.* **1969**, *30*, 289.
(233) Ugi, I. *Intra-Sci. Chem. Rep.* **1971**, *5*, 229.
(234) Gokel, G.; Hoffmann, P.; Kleimann, H.; Klusacek, H.; Lüdke, G.; Marquarding, D.; Ugi, I. In *Isonitrile Chemistry*; Ugi, I., Ed.; Academic Press: New York, 1971; p 201.
(235) Ugi, I.; Arora, A.; Burghard, H.; Eberle, G.; Eckert, H.; George, G.; Gokel, G.; Herlinger, H.; v. Hinrichs, E.; Hoffmann, P.; Kleimann, H.; Klusacek, H.; Lam, H.-L.; Marquarding, D.; Nah, H.-S.; Offermann, K.; Rehn, D.; Stüber, S.; Tamasi, M.; Urban, R.; Wackerle, L.; Zahr, S.; v. Zychlinski, H. In *Peptides 1974*; Wiley: New York, 1975; p 71.
(236) Ugi, I.; Steinbrückner, C. *Chem. Ber.* **1961**, *94*, 2802.
(237) Maia, H. L.; Ridge, B.; Rydon, H. N. *J. Chem. Soc., Perkin Trans. 1* **1973**, 98.
(238) Failli, A.; Sestanj, K.; Immer, H. U.; Götz, M. *Arzneim.-Forsch./Drug Res.* **1977**, *27(II)*, 2286.
(239) Failli, A.; Nelson, V.; Immer, H. U.; Götz, M. *Can. J. Chem.* **1973**, *51*, 2769.
(240) Immer, H. U.; Nelson, V.; Robinson, W.; Götz, M. *Liebigs Ann. Chem.* **1973**, 1789.
(241) Marquarding, D.; Hoffmann, P.; Heitzer, H.; Ugi, I. *J. Am. Chem. Soc.* **1970**, *92*, 1969.
(242) Urban, R.; Ugi, I. *Angew. Chem.* **1975**, *87*, 67; *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 4.
(243) Urban, R.; Eberle, G.; Marquarding, D.; Rehn, D.; Rehn, H.; Ugi, I. *Angew. Chem.* **1976**, *88*, 644; *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 627.
(244) Urban, R. *Tetrahedron* **1979**, *35*, 1841.
(245) Eberle, G.; Ugi, I. *Angew. Chem.* **1976**, *88*, 509; *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 492.
(246) Herrmann, R.; Hübener, G.; Siglmüller, F.; Ugi, I. *Liebigs Ann. Chem.* **1986**, 251.
(247) Siglmüller, F.; Herrmann, R.; Ugi, I. *Tetrahedron* **1986**, *42*, 5931.
(248) Urban, R.; Marquarding, D.; Ugi, I. *Hoppe-Seyler's Z. Physiol. Chem.* **1978**, *359*, 1541.
(249) Ratajczak, A.; Czech, B.; Misterkiewicz, B. *Bull. Acad. Pol. Sci., Ser. Sci. Chim.* **1977**, *25*, 541.
(250) Ratajczak, A.; Misterkiewicz, B. *J. Organomet. Chem.* **1975**, *91*, 73.
(251) French Patent 1595623, 1970; *Chem. Abstr.* **1971**, *75*, 6359u.
(252) U.S. Patent 3647775, 1972; *Chem. Abstr.* **1972**, *77*, 114886p.
(253) Ratajczak, A.; Czech, A. *Bull. Acad. Pol. Sci., Ser. Sci. Chim.* **1979**, *27*, 661.
(254) Pazur, J. H.; Kleppe, K.; Cepure, A. *Arch. Biochem. Biophys.* **1965**, *111*, 351.
(255) Kalisz, H. M.; Hecht, H. J.; Schomburg, D.; Schmid, R. D. *Biochim. Biophys. Acta* **1991**, *1080*, 138.
(256) Pazur, J. H.; Kleppe, K. *Biochemistry* **1964**, *3*, 578.
(257) Jones, M. N.; Manley, P.; Wilkinson, A. *Biochem. J.* **1982**, *203*, 285.
(258) Kriechbaum, M.; Heilmann, H. J.; Wientjes, F. J.; Hahn, M.; Jany, K.-D.; Gassen, H. G.; Sharif, F.; Alaeddinoglu, G. *FEBS Lett.* **1989**, *255*, 63.
(259) Frederick, K. R.; Tung, J.; Emerick, R. S.; Masiarz, F. R.; Chamberlain, S. H.; Vasavada, A.; Rosenberg, S.; Chakraborty, S.; Schopter, L. M.; Massey, V. *J. Biol. Chem.* **1990**, *265*, 3793.
(260) Kalisz, H. M.; Hecht, H. J.; Schomburg, D.; Schmid, R. D. *J. Mol. Biol.* **1990**, *213*, 207.
(261) Hecht, H. J.; Kalisz, H. M.; Hendle, J.; Schmid, R. D.; Schomburg, D. *J. Mol. Biol.* **1993**, *229*, 153.
(262) Wolowacz, S. E.; Yon Hin, B. F. Y.; Lowe, C. R. *Anal. Chem.* **1992**, *64*, 1541.
(263) European Patent Appl. 84303090.9, 1984; *Chem. Abstr.* **1984**, *102*, 128355m.
(264) Bartlett, P. N.; Whitaker, R. G.; Green, M. J.; Frew, J. *J. Chem. Soc., Chem. Commun.* **1987**, 1603.
(265) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285.
(266) Bartlett, P. N.; Bradford, V. Q.; Whitaker, R. G. *Talanta* **1991**, *38*, 57.
(267) Degani, Y.; Heller, A. *J. Am. Chem. Soc.* **1988**, *110*, 2615.
(268) Heller, A.; Degani, Y. In *Redox Chemistry and Interfacial Behaviour of Biological Molecules*; Dryhurst, G., Niki, K., Eds.; Plenum Publ. Corp.: New York, 1988; p 151.

(269) Schuhmann, W.; Ohara, T. J.; Schmidt, H.-L.; Heller, A. *J. Am. Chem. Soc.* **1991**, *113*, 1394.
(270) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128.
(271) Ryabov, A. D.; Trushkin, A. M.; Baksheeva, L. I.; Gorbatova, R. K.; Kubrakova, I. V.; Mozhaev, V. V.; Gnedenko, B. B.; Levashov, A. V. *Angew. Chem.* **1992**, *104*, 788; *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 789.
(272) Badia, A.; Carlini, R.; Fernandez, A.; Battaglini, F.; Mikkelsen, S. R.; English, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 7053.
(273) Blonder, R.; Katz, E.; Cohen, Y.; Itzhak, N.; Riklin, A.; Willner, I. *Anal. Chem.* **1996**, *68*, 3151.
(274) Kunugi, S.; Murakami, Y.; Ikeda, K.; Itoh, N. *Int. J. Biol. Macromol.* **1992**, *14*, 210.
(275) Blonder, R.; Ben-Dov, I.; Dagan, A.; Willner, I.; Zisman, E. *Biosens. Bioelectron.* **1997**, *12*, 627.
(276) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(277) Gray, H. B.; Winkler, J. R. *Annu. Rev. Biochem.* **1996**, *65*, 537.
(278) Nagata, R.; Yokoyama, K.; Clark, S. A.; Karube, I. *Biosens. Bioelectron.* **1995**, *10*, 261.
(279) Nagata, R.; Clark, S. A.; Yokoyama, K.; Tamiya, E.; Karube, I. *Anal. Chim. Acta* **1995**, *304*, 157.
(280) Nagata, R.; Yokoyama, K.; Durliat, H.; Comtat, M.; Clark, S. A.; Karube, I. *Electroanalysis* **1995**, *7*, 1027.
(281) Riklin, A.; Katz, E.; Willner, I.; Stocker, A.; Bückmann, A. F. *Nature* **1995**, *376*, 672.
(282) Katz, E.; Riklin, A.; Heleg-Shabtai, V.; Willner, I.; Bückmann, A. F. *Anal. Chim. Acta* **1999**, *385*, 45.
(283) Chen, L.-Q.; Zhang, X.-E.; Xie, W.-H.; Zhou, Y.-F.; Zhang, Z.-P.; Cass, A. E. G. *Biosens. Bioelectron.* **2002**, *17*, 851.
(284) Blonder, R.; Levi, S.; Tao, G.; Ben-Dov, I.; Willner, I. *J. Am. Chem. Soc.* **1997**, *119*, 10467.
(285) Willner, I.; Doron, A.; Katz, E.; Levi, S.; Frank, A. J. *Langmuir* **1996**, *12*, 946.
(286) Katz, E.; Willner, B.; Willner, I. *Biosens. Bioelectron.* **1997**, *12*, 703.
(287) Patolsky, F.; Filanovsky, B.; Katz, E.; Willner, I. *J. Phys. Chem. B* **1998**, *B102*, 10359.
(288) Willner, I.; Katz, E.; Willner, B. *Electroanalysis* **1997**, *9*, 965.
(289) Suzawa, T.; Ikariyama, Y.; Aizawa, M. *Anal. Chem.* **1994**, *66*, 3889.
(290) Krebs, H. A. *Biochem. J.* **1935**, *24*, 1620.
(291) Warburg, O.; Christian, W. *Biochem. Z.* **1938**, *298*, 150.
(292) Friedman, M. *J. Agric. Food Chem.* **1999**, *47*, 3457.
(293) Pilone, M. S. *Cell Mol. Life Sci.* **2000**, *57*, 1732.
(294) Curti, B.; Ronchi, S.; Branzoli, U.; Ferri, G.; Williams, C. H., Jr. *Biochim. Biophys. Acta* **1973**, *327*, 266.
(295) Simonetta, M. P.; Vanoni, M. A.; Casalin, P. *Biochim. Biophys. Acta* **1987**, *914*, 136.
(296) Pollegioni, L.; Buto, S.; Tischer, W.; Ghisla, S.; Pilone, M. S. *Biochem. Mol. Biol. Int.* **1993**, *31*, 709.
(297) Pilone Simonetta, M.; Pollegioni, L.; Casalin, P.; Curti, B.; Ronchi, S. *Eur. J. Biochem.* **1989**, *180*, 190.
(298) Mattevi, A.; Vanoni, M. A.; Todone, F.; Rizzi, M.; Teplyakov, A.; Coda, A.; Bolognesi, M.; Curti, B. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 7496.
(299) Todone, F.; Vanoni, M. A.; Mozzarelli, A.; Bolognesi, M.; Coda, A.; Curti, B.; Mattevi, A. *Biochemistry* **1997**, *36*, 5853.
(300) Mizutani, H.; Miyahara, I.; Hirotsu, K.; Nishina, Y.; Shiga, K.; Setoyama, C.; Miura, R. *J. Biochem.* **1996**, *120*, 14.
(301) Miura, R.; Setoyama, C.; Nishina, Y.; Shiga, K.; Mizutani, H.; Miyahara, I.; Hirotsu, K. *J. Biochem.* **1997**, *122*, 825.
(302) Mizuno, T.; Miyahara, I.; Hirotsu, K.; Nishina, Y.; Shiga, K.; Setoyama, C.; Miura, R. *J. Biochem.* **2000**, *128*, 73.
(303) Umhau, S.; Pollegioni, L.; Molla, G.; Diederichs, K.; Welte, W.; Pilone, M. S.; Ghisla, S. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 12463.
(304) Pollegioni, L.; Diederichs, K.; Molla, G.; Umhau, S.; Welte, W.; Ghisla, S.; Pilone, M. S. *J. Mol. Biol.* **2002**, *324*, 535.
(305) Gelb, M. H.; Heimbrook, D. C.; Mälkönen, P.; Sligar, S. G. *Biochemistry* **1982**, *21*, 370.
(306) Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122.
(307) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314.
(308) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687.
(309) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 917.
(310) Raag, R.; Poulos, T. L. *Biochemistry* **1991**, *30*, 2674.
(311) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 7586.
(312) Poulos, T. L.; Howard, A. J. *Biochemistry* **1987**, *26*, 8165.
(313) Raag, R.; Li, H.; Jones, B. C.; Poulos, T. L. *Biochemistry* **1993**, *32* (2), 4571.
(314) Mueller, E. J.; Loida, P. J.; Sligar, S. G. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; p 83.
(315) Di Gleria, K.; Nickerson, D. P.; Hill, H. A. O.; Wong, L.-L.; Fülöp, V. *J. Am. Chem. Soc.* **1998**, *120*, 46.
(316) Lo, K.-W. K.; Wong, L.-L.; Hill, H. A. O. *FEBS Lett.* **1999**, *451*, 342.
(317) Kostic, N. M. *Comments Inorg. Chem.* **1988**, *8*, 137.
(318) Lukes, A. J.; Kostic, N. M. *J. Inorg. Biochem.* **1992**, *46*, 77.
(319) Sünkel, K.; Kiessling, T. *J. Organomet. Chem.* **2001**, *637−639*, 796.
(320) Welinder, K. G. *Eur. J. Biochem.* **1979**, *96*, 483.
(321) Shiro, Y.; Kurono, M.; Morishima, I. *J. Biol. Chem.* **1986**, *261*, 9382.
(322) Smith, A. T.; Santama, N.; Dacey, S.; Edwards, M.; Bray, R. C.; Thorneley, R. N. F.; Burke, J. F. *J. Biol. Chem.* **1990**, *265*, 13335.
(323) Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. *Nature Struct. Biol.* **1997**, *4*, 1032.
(324) Goral, V. N.; Ryabov, A. D. *Biochem. Mol. Biol. Int.* **1998**, *45*, 61.
(325) Ryabov, A. D.; Goral, V. N. *J. Biol. Inorg. Chem.* **1997**, *2*, 182.
(326) Frew, J. E.; et al. *J. Electroanal. Chem.* **1986**, *210*, 1.
(327) Smit, M. H.; Cass, A. E. G. *Anal. Chem.* **1990**, *62*, 2429.
(328) Vidal, J. C.; Yague, M. A.; Castillo, J. R. *Sens. Actuators* **1994**, *B21*, 135.
(329) Tsai, W.-C.; Cass, A. E. G. *Analyst* **1995**, *120*, 2249.
(330) Ryabov, A. D.; Goral, V. N.; Gorton, L.; Csöregi, E. *Chem. Eur. J.* **1999**, *5*, 961.
(331) Gill, T. J., III; Mann, L. T., Jr. *J. Immunol.* **1966**, *96*, 906.
(332) Mann, L. T., Jr. *J. Labelled Compd. Radiopharm.* **1967**, *3*, 87.
(333) Carpenter, C. B.; Gill, T. J., III; Mann, L. T., Jr. *J. Immunol.* **1967**, *98*, 236.
(334) Peterlik, M. *Monatsh. Chem.* **1967**, *98*, 2133.
(335) Franz, H. *Z. Chem.* **1967**, *7*, 235.
(336) Franz, H. *Z. Chem.* **1967**, *7*, 427.
(337) Franz, H. *Naturwissenschaften* **1967**, *54*, 339.
(338) Franz, H.; Scheuner, G. *Histochemie* **1968**, *16*, 159.
(339) Franz, H. *Histochemie* **1968**, *16*, 220.
(340) Allen, D. E.; Perrin, D. D. *J. Histochem. Cytochem.* **1974**, *22*, 919.
(341) Swift, A. J. *J. Histochem. Cytochem.* **1975**, *23*, 390.
(342) Wildführ, W.; Mehner, E. *Z. Gesamte Hyg. Ihre Grenzgeb.* **1969**, *15*, 616.
(343) Wagner, B.; Wagner, M. *Zbl. Bakt. Hyg., I. Abt. Orig.* **1972**, *A222*, 468.
(344) Franz, H.; Wildführ, W. *Z. Immunitaetsforsch., Exp. Klin. Immunol.* **1971**, *142*, 334.
(345) Micheel, B.; Bierwolf, D.; Randt, A.; Franz, H.; Mohr, J.; Pasternak, G. *Acta Biol. Med. Ger.* **1971**, *27*, 639.
(346) Wildführ, W.; Franz, H. *Zbl. Bakt. Hyg., I. Abt. Orig.* **1971**, *216*, 532.
(347) Franz, H.; Mohr, J. *Z. Inn. Med.* **1969**, *24*, 673.
(348) Franz, H. *Histochemie* **1968**, *12*, 230.
(349) Yasuda, K.; Yamamoto, N. *Acta Histochem. Cytochem.* **1975**, *8*, 215.
(350) Lim, T.-K.; Nakamura, N.; Ikehata, M.; Matsunaga, T. *Electrochemistry* **2000**, *11*, 872.
(351) Kossek, S.; Padeste, C.; Tiefenauer, L. *J. Mol. Recognit.* **1996**, *9*, 485.
(352) Tiefenauer, L. X.; Kossek, S.; Padeste, C.; Thiébaud, P. *Biosens. Bioelectron.* **1997**, *12*, 213.
(353) Wang, J.; Ibánez, A.; Chatrathi, M. P. *Electrophoresis* **2002**, *23*, 3744.
(354) Lim, T.-K.; Ohta, H.; Matsunaga, T. *Anal. Chem.* **2003**, *75*, 3316.
(355) Lim, T.-K.; Imai, S.; Matsunaga, T. *Biotechnol. Bioeng.* **2002**, *77*, 758.
(356) Lim, T.-K.; Matsunaga, T. *Biosens. Bioelectron.* **2001**, *16*, 1063.
(357) Choi, M. J.; Kim, S. Y.; Choi, J.; Paeng, I. R. *Microchem. J.* **1999**, *63*, 92.
(358) Yasuda, K. *J. Electron Microsc.* **1978**, *27*, 361.
(359) Anne, A.; Demaille, C.; Moiroux, J. *J. Am. Chem. Soc.* **1999**, *121*, 10379.
(360) Anne, A.; Demaille, C.; Moiroux, J. *J. Am. Chem. Soc.* **2001**, *123*, 4817.
(361) Demaille, C.; Moiroux, J. *J. Phys. Chem.* **1999**, *B103*, 9903.
(362) Pubmed Entry No. CAA76847.
(363) Falk, H.; Peterlik, M.; Schlögl, K. *Monatsh. Chem.* **1969**, *100*, 787.
(364) Badia, A.; Thai, N. H. H.; English, A. M.; Mikkelsen, S. R.; Patterson, R. T. *Anal. Chim. Acta* **1992**, *262*, 87.
(365) Mizutani, F.; Asai, M. *Denki Kagaku* **1988**, *56*, 1100.
(366) Mizutani, F.; Asai, M. In *Proceedings of the MRS International Meeting on Advanced Materials*; Doyama, M., Somiya, S., Chang, R. P. H., Karube, I., Eds.; MRS Materials Research Society: Pittsburgh, PA, 1989; Vol. 14, p 147.
(367) Shinohara, H.; Kusaka, T.; Yokota, E.; Monden, R.; Sisido, M. *Sens. Actuators* **2000**, *B65*, 144.
(368) Suzawa, T.; Ikariyama, Y.; Aizawa, M. *Bull. Chem. Soc. Jpn.* **1995**, *68*, 165.
(369) Taborsky, G. *Adv. Protein Chem.* **1974**, *28*, 1.
(370) McReynolds, L.; O'Malley, B. W.; Nisbet, A. D.; Fothergill, J. E.; Givol, D.; Fields, S.; Robertson, M.; Brownlee, G. G. *Nature* **1978**, *273*, 723.
(371) Nisbet, A. D.; Saundry, R. I.; Moir, A. J. G.; Fothergill, L. A.; Fothergill, J. E. *Eur. J. Biochem.* **1981**, *115*, 335.
(372) Narita, K.; Ishii, J. *J. Biochem.* **1962**, *52*, 367.

(373) Stein, P. E.; Leslie, A. G. W.; Finch, J. T.; Carrell, R. W. *J. Mol. Biol.* **1991**, *221*, 941.
(374) Lowe, G. *Tetrahedron* **1976**, *32*, 291.
(375) Husain, S. S.; Lowe, G. *Biochem. J.* **1969**, *114*, 279.
(376) Husain, S. S.; Lowe, G. *Biochem. J.* **1970**, *116*, 689.
(377) Mitchel, R. E. J.; Chaiken, I. M.; Smith, E. L. *J. Biol. Chem.* **1970**, *245*, 3485.
(378) Drenth, J.; Jansonius, J. N.; Koekoek, R.; Swen, H. M.; Wolthers, B. G. *Nature* **1968**, *218*, 929.
(379) Douglas, K. T.; Ejim, O. S.; Taylor, K. *J. Enzyme Inhib.* **1992**, *6*, 233.
(380) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85.
(381) Chaiet, L.; Wolf, F. J. *Arch. Biochem. Biophys.* **1964**, *106*, 1.
(382) Argarana, C. E.; Kuntz, I. D.; Birken, S.; Axel, R.; Cantor, C. R. *Nucleic Acids Res.* **1986**, *14*, 1871.
(383) Wilchek, M.; Bayer, E. A. *Trends Biochem. Sci.* **1989**, *14*, 408.
(384) Bayer, E. A.; Wilchek, M. *J. Chromatogr.* **1990**, *510*, 3.
(385) Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5076.
(386) Pugliese, L.; Coda, A.; Malcovati, M.; Bolognesi, M. *J. Mol. Biol.* **1993**, *231*, 698.
(387) Hendrickson, W. A.; Pähler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackerley, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190.
(388) Weber, P. C.; Ohlendorf, D. H.; Wendolowski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85.
(389) Padeste, C.; Grubelnik, A.; Tiefenauer, L. *Biosens. Bioelectron.* **2000**, *15*, 431.
(390) Steiger, B.; Padeste, C.; Grubelnik, A.; Tiefenauer, L. *Electrochim. Acta* **2003**, *48*, 761.
(391) Mosbach, M.; Schuhmann, W. *Sens. Actuators* **2000**, *B70*, 145.
(392) Mironova, E. V.; Lukin, A. Y.; Shevyakov, S. V.; Alexeeva, S. G.; Shvets, V. I.; Demina, O. V.; Khodonov, A. A.; Khitrina, L. V. *Biochemistry (Moscow)* **2001**, *66*, 1323; translation from *Biokhimiya* **2001**, *66*, 1638.
(393) Oesterhelt, D.; Stoeckenius, W. *Nature New Biol.* **1971**, *233*, 149.
(394) Hampp, N. *Chem. Rev.* **2000**, *100*, 1755.
(395) Haupts, U.; Tittor, J.; Oesterhelt, D. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 367.
(396) Findlay, J. B. C.; Pappin, D. J. C. *Biochem. J.* **1986**, *238*, 625.
(397) Wilson, E. K. *Chem. Eng. News* **1969**, *47*, 7.
(398) Beilstein, A. E.; Tierney, M. T.; Grinstaff, M. W. *Comments Inorg. Chem.* **2000**, *22*, 105.
(399) Hu, X.; Lee, S. S.; Grinstaff, M. W. *Methods Enzymol.* **2002**, *353*, 548.
(400) Meunier, P.; Ouattara, I.; Gautheron, B.; Tirouflet, J.; Camboli, D.; Besancon, J.; Boulay, F. *Eur. J. Med. Chem.* **1991**, *26*, 351.
(401) Yu, C. J.; Yowanto, H.; Wan, Y.; Meade, T. J.; Chong, Y.; Strong, M.; Donilon, L. H.; Kayyem, J. F.; Gozin, M.; Blackburn, G. F. *J. Am. Chem. Soc.* **2000**, *122*, 6767.
(402) Coutouli-Argyropoulou, E.; Tsitabani, M.; Petrantonakis, G.; Terzis, A.; Raptopoulou, C. *Org. Biomol. Chem.* **2003**, *1*, 1382.
(403) Robins, M. J.; Barr, P. J. *Tetrahedron Lett.* **1981**, 421.
(404) Robins, M. J.; Barr, P. J. *J. Org. Chem.* **1983**, *48*, 1854.
(405) Robins, M. J.; Vinayak, R. S.; Wood, S. G. *Tetrahedron Lett.* **1990**, *31*, 3731.
(406) McGuigan, C.; Yarnold, C. J.; Jones, G.; Velázquez, S.; Baruchi, H.; Brancale, A.; Andrei, G.; Snoeck, R.; De Clercq, E.; Balzarini, E. *J. Med. Chem.* **1999**, *42*, 4479.
(407) Appel, A.; Jäkle, F.; Priermeier, T.; Schmid, R.; Wagner, M. *Organometallics* **1996**, *15*, 1188.
(408) Pike, A. R.; Ryder, L. C.; Horrocks, B. R.; Clegg, W.; Elsegood, M. R. J.; Connolly, B. A.; Houlton, A. *Chem. Eur. J.* **2002**, *8*, 2891.
(409) Price, C.; Aslanoglu, M.; Isaac, C. J.; Elsegood, M. R. J.; Clegg, W.; Horrocks, B. R.; Houlton, A. *J. Chem. Soc., Dalton Trans.* **1996**, 4115.
(410) Houlton, A.; Isaac, C. J.; Gibson, A. E.; Horrocks, B. R.; Clegg, W.; Elsegood, M. R. J. *J. Chem. Soc., Dalton Trans.* **1999**, 3229.
(411) Chen, S.-C. *J. Organomet. Chem.* **1980**, *202*, 183.
(412) Inouye, M.; Takase, M. *Angew. Chem.* **2001**, *113*, 1796; *Angew. Chem., Int. Ed* **2001**, *40*, 1746.
(413) Glass, R. S.; Stessman, N. Y. T. *Tetrahedron Lett.* **2000**, *41*, 9581.
(414) Bucci, E.; De Napoli, L.; Di Fabio, G.; Messere, A.; Montesarchio, D.; Romanelli, A.; Picciallia, G.; Varra, M. *Tetrahedron* **1999**, *55*, 14435.
(415) Jacobson, K. B.; Arlinghaus, H. F.; Schmitt, H. W.; Sachleben, R. A.; Brown, G. M.; Thonnard, N.; Slopp, F. V.; Foote, R. S.; Larimer, F. W.; Woychik, R. P.; England, M. W.; Burchett, K. L.; Jacobson, D. A. *Genomics* **1991**, *9*, 51.
(416) Arlinghaus, H. F.; Thonnard, N.; Spaar, M. T.; Sachleben, R. A.; Larimer, F. W.; Foote, R. S.; Woychik, R. P.; Brown, G. M.; Slopp, F. V.; Jacobson, K. B. *Anal. Chem.* **1991**, *63*, 402.
(417) Slopp, F. V.; Brown, G. M.; Sachleben, R. A.; Garrity, M. L.; Elbert, J. E.; Jacobson, K. B. *New J. Chem.* **1994**, *18*, 317.
(418) Beilstein, A. E.; Grinstaff, M. W. *J. Chem. Soc., Chem. Commun.* **2000**, 509.
(419) Beilstein, A. E.; Grinstaff, M. W. *J. Organomet. Chem.* **2001**, *637−639*, 398.
(420) Khan, S. I.; Grinstaff, M. W. *J. Am. Chem. Soc.* **1999**, *121*, 4704.
(421) Khan, S. I.; Beilstein, A. E.; Tierney, M. T.; Sykora, M.; Grinstaff, M. W. *Inorg. Chem.* **1999**, *38*, 5999.
(422) Anne, A.; Blanc, B.; Moiroux, J. *Bioconjugate Chem.* **2001**, *12*, 396.
(423) Wlassoff, W. A.; King, G. C. *Nucleic Acids Res.* **2002**, *30*, e58.
(424) Okamoto, A.; Tainaka, K.; Saito, I. *Tetrahedron Lett.* **2002**, *43*, 4581.
(425) Stryer, L. *Biochemistry*, 4th ed.; W. H. Freeman and Company: New York, 1994.
(426) Pividori, M. I.; Merkoci, A.; Alegret, S. *Biosens. Bioelectron.* **2000**, *15*, 291.
(427) Palcek, E.; Fojta, M. *Anal. Chem.* **2001**, *73*, 75a.
(428) McGlennen, R. C. *Clin. Chem.* **2001**, *47*, 393.
(429) Pike, A. R.; Horrocks, B.; Connolly, B.; Houlton, A. *Aust. J. Chem.* **2002**, *55*, 191.
(430) Lacher, N. A.; Garrison, K. E.; Martin, R. S.; Lunte, S. M. *Electrophoresis* **2001**, *22*, 2526.
(431) Farkas, D. H. *Clin. Chem.* **2001**, *47*, 1871.
(432) Umek, R. M.; Lin, S. S.; Chen, Y.-P.; Irvine, B.; Paulluconi, G.; Chan, V.; Chong, Y.; Cheung, L.; Vielmetter, J.; Farkas, D. H. *Mol. Diagn.* **2000**, *5*, 321.
(433) Wang, J.; Rivas, G.; Fernandes, J. R.; Paz, J. L. L.; Jiang, M.; Waymire, R. *Anal. Chim. Acta* **1998**, *375*, 197.
(434) Takenaka, S.; Uto, Y.; Kondo, H.; Ihara, T.; Takagi, M. *Anal. Biochem.* **1994**, *218*, 436.
(435) Takenaka, S.; Yamashita, K.; Takagi, M.; Uto, Y.; Kondo, H. *Anal. Chem.* **2000**, *72*, 1334.
(436) Takenaka, S. *Bull. Chem. Soc. Jpn.* **2001**, *74*, 217.
(437) Ihara, T.; Maruo, Y.; Takenaka, S.; Takagi, M. *Nucleic Acids Res.* **1996**, *24*, 4273.
(438) Beck, S.; Köster, H. *Anal. Chem.* **1990**, *62*, 2258.
(439) Petraccone, L.; Erra, E.; Messere, A.; Montesarchio, D.; Piccialli, G.; Barone, G.; Giancola, C. *Biophys. Chem.* **2003**, *104*, 259.
(440) Uto, Y.; Kondo, H.; Abe, M.; Suzuki, T.; Takenaka, S. *Anal. Biochem.* **1997**, *250*, 122.
(441) Brazill, S. A.; Kim, P. H.; Kuhr, W. G. *Anal. Chem.* **2001**, *73*, 4882.
(442) Brazill, S. A.; Kuhr, W. G. *Anal. Chem.* **2002**, *74*, 3421.
(443) Brazill, S. A.; Singhal, P.; Kuhr, W. G. *Anal. Chem.* **2000**, *72*, 5542.
(444) Mucic, R. C.; Herrlein, M. K.; Mirkin, C. A.; Letsinger, R. L. *J. Chem. Soc., Chem. Commun.* **1996**, 555.
(445) Long, Y.-T.; Li, C.-Z.; Sutherland, T. C.; Chahma, M.; Lee, J. S.; Kraatz, H.-B. *J. Am. Chem. Soc.* **2003**, *125*, 8724.
(446) Chahma, M.; Lee, J. S.; Kraatz, H.-B. *J. Electroanal. Chem.* **2004**, *567*, 283.
(447) Anne, A.; Bouchardon, A.; Moiroux, J. *J. Am. Chem. Soc.* **2003**, *125*, 1112.
(448) Fan, C.; Plaxco, K. W.; Heeger, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9134.
(449) Kim, K.; Yang, H.; Park, S. H.; Lee, D.-S.; Kim, S.-J.; Lim, Y. T.; Kim, Y. T. *J. Chem. Soc., Chem. Commun.* **2004**, 1466.
(450) Ihara, T.; Nakayama, M.; Murata, M.; Nakano, K.; Maeda, M. *J. Chem. Soc., Chem. Commun.* **1997**, 1609.
(451) Nakayama, M.; Ihara, T.; Nakano, K.; Maeda, M. *Talanta* **2002**, *56*, 857.
(452) Yu, C. J.; Wang, H.; Wan, Y.; Yowanto, H.; Kim, J. C.; Donilon, L. H.; Tao, C.; Strong, M.; Cong, Y. *J. Org. Chem.* **2001**, *66*, 2937.
(453) Umek, R. M.; Lin, S. W.; Vielmetter, J.; Terbrueggen, R. H.; Irvine, B.; Yu, C. J.; Kayyem, J. F.; Yowanto, H.; Blackburn, G. F.; Farkas, D. H.; Chen, Y.-P. *J. Mol. Diagn.* **2001**, *3*, 74.
(454) Yu, C. J.; Wan, Y.; Yowanto, H.; Li, J.; Tao, C.; James, M. D.; Tan, C. L.; Blackburn, G. F.; Meade, T. J. *J. Am. Chem. Soc.* **2001**, *123*, 11155.
(455) Di Giusto, D. A.; Wlassoff, W. A.; Giesebrecht, S.; Gooding, J. J.; King, G. C. *Angew. Chem.* **2004**, *116*, 2869; *Angew. Chem., Int. Ed.* **2004**, *43*, 2809.
(456) Xu, C.; Cai, H.; He, P.; Fang, Y. *Fresenius J. Anal. Chem.* **2000**, *367*, 593.
(457) Xu, C.; He, P.; Fang, Y. *Anal. Chim. Acta* **2000**, *411*, 31.
(458) Xu, C.; Cai, H.; He, P.; Fang, Y. *Analyst* **2001**, *126*, 62.
(459) Creager, S.; Yu, C. J.; Bamdad, C.; O'Connor, S.; MacLean, T.; Lam, E.; Chong, Y.; Olsen, G. T.; Luo, J.; Gozin, M.; Kayyem, J. F. *J. Am. Chem. Soc.* **1999**, *121*, 1059.
(460) Pike, A. R.; Lie, L. H.; Eagling, R. A.; Ryder, L. C.; Patole, S. N.; Connolly, B. A.; Horrocks, B. R.; Houlton, A. *Angew. Chem.* **2002**, *114*, 637; *Angew. Chem., Int. Ed.* **2002**, *41*, 615.
(461) Cloarec, J. P.; Martin, J. R.; Polychronakos, C.; Lawrence, I.; Lawrence, M. F.; Souteyrand, E. *Sens. Actuators, B: Chem.* **1999**, *58*, 394.
(462) Cloarec, J. P.; Deligianis, N.; Martin, J. R.; Lawrence, I.; Souteyrand, E.; Polychronakos, C.; Lawrence, M. F. *Biosens. Bioelectron.* **2002**, *17*, 405.
(463) Marquette, C. A.; Lawrence, I.; Polychronakos, C.; Lawrence, M. F. *Talanta* **2002**, *56*, 763.
(464) Boukherroub, R.; Wayner, D. D. M. *J. Am. Chem. Soc.* **1999**, *121*, 11513.

(465) Lopinski, G. P.; Wayner, D. D. M.; Wolkow, R. A. *Nature* **2000**, *406*, 48.
(466) Takenaka, S.; Yamashita, K.; Takagi, M.; Uto, Y.; Kondo, H. *Anal. Chem.* **2000**, *72*, 1334.
(467) Yamashita, K.; Takagi, M.; Kondo, H.; Takenaka, S. *Chem. Lett.* **2000**, 1038.
(468) Takenaka, S.; Uto, Y.; Takagi, M.; Kondo, H. *Chem. Lett.* **1998**, 989.
(469) Yamashita, K.; Takagi, A.; Takagi, M.; Kondo, H.; Ikeda, K.; Takenaka, S. *Bioconjugate Chem.* **2002**, *13*, 1193.
(470) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497.
(471) Nielsen, P. E. *Acc. Chem. Res.* **1999**, *32*, 624.
(472) Uhlmann, E.; Peyman, A.; Breipohl, G.; Will, D. W. *Angew. Chem.* **1998**, *110*, 2954; *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2796.
(473) Dueholm, K. L.; Engholm, M.; Behrens, C.; Christensen, L.; Hansen, H. F.; Vulpius, T.; Petersen, K. H.; Berg, R. H.; Nielsen, P. E.; Buchardt, O. *J. Org. Chem.* **1994**, *59*, 5767.
(474) Thomson, S. A.; Josey, J. A.; Cadilla, R.; Gaul, M. D.; Hassman, C. F.; Luzzio, M. J.; Pipe, A. J.; Reed, K. L.; Ricca, D. J.; Wiethe, R. W.; Noble, S. A. *Tetrahedron* **1995**, *51*, 6179.
(475) Egholm, M.; Buchardt, O.; Christensen, L.; Behrens, C.; Freier, S. M.; Driver, D. A.; Berg, R. H.; Kim, S. K.; Norden, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566.
(476) Leijon, M.; Gräslund, A.; Nielsen, P. E.; Buchardt, O.; Nordén, B.; Kristensen, S. M.; Eriksson, M. *Biochemistry* **1994**, *33*, 9820.
(477) Brown, S. C.; Thomson, S. A.; Veal, J. M.; Davis, D. G. *Science* **1994**, *265*, 777.
(478) Ratilainen, T.; Holmén, A.; Tuite, E.; Haaima, G.; Christensen, L.; Nielsen, P. E.; Nordén, B. *Biochemistry* **1992**, *31*, 12331.
(479) Ratilainen, T.; Holmén, A.; Tuite, E.; Nielsen, P. E.; Nordén, B. *Biochemistry* **2000**, *39*, 7781.
(480) Eriksson, M.; Nielsen, P. E. *Q. Rev. Biophys.* **1996**, *29*, 369.
(481) Larsen, H. J.; Bentin, T.; Nielsen, P. E. *Biochim. Biophys. Acta* **1999**, *1489*, 159.
(482) Verheijen, J. C.; van der Marel, G. A.; van Boom, J. H.; Metzler-Nolte, N. *Bioconjugate Chem.* **2000**, *11*, 741.
(483) de Belder, A. N.; Bourne, E. J.; Pridham, J. B. *J. Chem. Soc.* **1961**, 4464.
(484) Collins, P. M.; Overend, W. G.; Rayner, B. A. *J. Chem. Soc., Perkin Trans. 2* **1973**, 310.
(485) Jones, C. C.; Sinnott, M. L.; Souchard, I. J. L. *J. Chem. Soc., Perkin Trans. 2* **1977**, 1191.
(486) Adam, M. J.; Hall, L. D. *J. Chem. Soc., Chem. Commun.* **1979**, 865.
(487) Adam, M. J.; Hall, L. D. *Can. J. Chem.* **1980**, *58*, 1188.
(488) Landells, J. S.; Kerr, J. L.; Larsen, D. S.; Robinson, B. H.; Simpson, J. *J. Chem. Soc., Dalton Trans.* **2000**, 1403.
(489) Kerr, J. L.; Landells, J. S.; Larsen, D. S.; Robinson, B. H.; Simpson, J. *J. Chem. Soc., Dalton Trans.* **2000**, 1411.
(490) Schneider, M.; Wenzel, M. *J. Labelled Compd. Radiopharm.* **1979**, *XVIII*, 293.
(491) Grynkiewicz, G.; BeMiller, J. N. *Carbohydr. Res.* **1984**, *131*, 273.
(492) Adam, M. J.; Hall, L. D. *J. Organomet. Chem.* **1980**, *186*, 289.
(493) Chahma, M.; Lee, J. S.; Kraatz, H.-B. *J. Organomet. Chem.* **2002**, *648*, 81.
(494) Shirakami, S.; Itoh, T. *Tetrahedron: Asymmetry* **2000**, *11*, 2823.
(495) Takeuchi, M.; Mizuno, T.; Shinkai, S.; Shirakami, S.; Itoh, T. *Tetrahedron: Asymmetry* **2000**, *11*, 3311.
(496) Itoh, T.; Shirakami, S. *J. Synth. Org. Chem. Jpn.* **2002**, *60*, 232.
(497) Itoh, T.; Shirakami, S.; Nakao, Y.; Yoshida, T. *Chem. Lett.* **1998**, 979.
(498) Itoh, T.; Shirakami, S.; Ishida, N.; Yamashita, Y.; Yoshida, T.; Kim, H.-S.; Wataya, Y. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1657.
(499) Hartinger, C. G.; Nazarov, A. A.; Arion, V. B.; Giester, G.; Jakupec, M.; Galanski, M.; Keppler, B. K. *New J. Chem.* **2002**, *26*, 671.
(500) Ashton, P. R.; Balzani, V.; Clemente-Leon, M.; Colonna, B.; Credi, A.; Jayaraman, N.; Raymo, F. M.; Stoddart, J. F.; Venturi, M. *Chem. Eur. J.* **2002**, *8*, 673.
(501) Dexter, D. L. *J. Chem. Phys.* **1953**, *21*, 836.
(502) Matsue, T.; Evans, D. H.; Osa, T.; Kobayashi, N. *J. Am. Chem. Soc.* **1985**, *107*, 3411.
(503) Isnin, R.; Salam, C.; Kaifer, A. E. *J. Org. Chem.* **1991**, *56*, 35.
(504) Kobayashi, N.; Opallo, M. *J. Chem. Soc., Chem. Commun.* **1990**, 477.
(505) Ueno, A.; Moriwaki, F.; Matsue, T.; Osa, T. *Makromol. Chem., Rapid Commun.* **1985**, *6*, 231.
(506) Ueno, A.; Suzuki, I.; Osa, T. *Makromol. Chem., Rapid Commun.* **1987**, *8*, 131.
(507) Suzuki, I.; Chen, Q.; Ueno, A.; Osa, T. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 1472.
(508) Menger, F. M.; Sherrod, M. J. *J. Am. Chem. Soc.* **1988**, *110*, 8606.
(509) Thiem, H.-J.; Brandl, M.; Breslow, R. *J. Am. Chem. Soc.* **1988**, *110*, 8612.
(510) Togni, A. *Angew. Chem.* **1996**, *108*, 1581; *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1475.
(511) Albinati, A.; Pregosin, P. S.; Wick, K. *Organometallics* **1996**, *15*, 2419.
(512) Vedso, P.; Chauvin, R.; Li, Z.; Bernet, B.; Vasella, A. *Helv. Chim. Acta* **1994**, *77*, 1631.
(513) Vasella, A.; Li, Z. *Helv. Chim. Acta* **1996**, *79*, 2201.
(514) Widauer, C.; Bernet, B.; Vasella, A. *Synth. Commun.* **1998**, *28*, 593.
(515) Riesselmann, B.; Wenzel, M. *Hoppe-Seyler's Z. Physiol. Chem.* **1977**, *358*, 1353.
(516) Cais, M.; Dani, S.; Eden, Y.; Gandolfi, O.; Horn, M.; Isaacs, E. E.; Josephy, Y.; Saar, Y.; Slovin, E.; Snarsky, L. *Nature* **1977**, *270*, 534.
(517) Cais, M. *L'actualite chimique* **1979**, *September*, 14.
(518) Salmain, M.; Vessières, A.; Brossier, P.; Butler, I. S.; Jaouen, G. *J. Immun. Methods* **1992**, *148*, 65.
(519) Vessières, A.; Salmain, M.; Brossier, P.; Jaouen, G. *J. Pharm. Biomed. Anal.* **1999**, *21*, 625.
(520) Vessières, A.; Vaillant, C.; Gruselle, M.; Vichard, D.; Jaouen, G. *J. Chem. Soc., Chem. Commun.* **1990**, 837.
(521) Vichard, D.; Gruselle, M.; Jaouen, G.; Nefedova, M. N.; Mamedyarova, I. A.; Sokolov, V. I.; Vaissermann, J. *J. Organomet. Chem.* **1994**, *484*, 1.
(522) Osella, D.; Gambino, O.; Dutto, G. C.; Nervi, C.; Jaouen, G.; Vessières, A. *Inorg. Chim. Acta* **1994**, *218*, 207.
(523) Wenzel, M.; Klinge, C. *J. Labelled Compd. Radiopharm.* **1994**, *34*, 981.
(524) Osella, D.; Nervi, C.; Galeotti, F.; Cavigiolio, G.; Vessières, A.; Jaouen, G. *Helv. Chim. Acta* **2001**, *84*, 3289.
(525) Jie, T.; Xiao-Guang, L. *Chin. J. Org. Chem.* **1999**, *19*, 312.
(526) Green, S.; Walter, P.; Kumar, V.; Krust, A.; Bornert, J.-M.; Argos, P.; Chambon, P. *Nature* **1986**, *320*, 134.
(527) Kuiper, G. G. J. M.; Enmark, E.; Pelto-Huikko, M.; Nilsson, S.; Gustafsson, J.-A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5925.
(528) Hoffmann, K.; Riesselmann, B.; Wenzel, M. *Liebigs Ann. Chem.* **1980**, 1181.
(529) Cais, M.; Slovin, E.; Snarsky, L. *J. Organomet. Chem.* **1978**, *160*, 223.
(530) Krieg, R.; Wyrwa, R.; Möllmann, U.; Görls, H.; Schönecker, B. *Steroids* **1998**, *63*, 531.
(531) Tang, J.; Top, S.; Vessières, A.; Sellier, N.; Vaissermann, J.; Jaouen, G. *Appl. Organomet. Chem.* **1997**, *11*, 771.
(532) El Amouri, H.; Vessières, A.; Vichard, D.; Top, S.; Gruselle, M.; Jaouen, G. *J. Med. Chem.* **1992**, *35*, 3130.
(533) Top, S.; El Hafa, H.; Vessières, A.; Quivy, J.; Vaissermann, J.; Hughes, D. W.; McGlinchey, M. J.; Mornon, J.-P.; Thoreau, E.; Jaouen, G. *J. Am. Chem. Soc.* **1995**, *117*, 8372.
(534) Vessières, A.; Vaillant, C.; Salmain, M.; Jaouen, G. *J. Steroid Biochem.* **1989**, *34*, 301.
(535) Vessières, A.; Jaouen, G.; Gruselle, M.; Rossignol, J. L.; Savignac, M.; Top, S.; Greenfield, S. *J. Steroid Biochem.* **1988**, *30*, 301.
(536) Shimada, K.; Nagashima, E.; Orii, S.; Nambara, T. *J. Pharm. Biomed. Anal.* **1987**, *5*, 361.
(537) Shimada, K.; Orii, S.; Tanaka, M.; Nambara, T. *J. Chromatogr.* **1986**, *352*, 329.
(538) Heydenhauss, D.; Jaenecke, G.; Schubert, H. *Z. Chem.* **1973**, *13*, 295.
(539) Koller, A. *Diss. Abstr. Int., B* **1975**, *35*, 5820.
(540) Robinson, G. A.; Martinazzo, G.; Forrest, G. C. *J. Immunoassay* **1986**, *7*, 1.
(541) Yao, T.; Rechnitz, G. A. *Biosensors* **1988**, *3*, 307.
(542) Shimada, K.; Sakayori, C.; Nambara, T. *J. Liq. Chromatogr.* **1987**, *10*, 2177.
(543) Troitskaya, L. L.; Sokolov, V. I. *Russ. Chem. Bull.* **1985**, *34*, 1548; translation from *Izv. Akad. Nauk, Ser. Khim.* **1985**.
(544) Troitskaya, L. L.; Sokolov, V. I. *J. Organomet. Chem.* **1985**, *285*, 389.
(545) Kim, W. H. *Diss. Abstr. Int., B* **1978**, *39*, 1774.
(546) Forrow, N. J.; Foulds, N. C.; Frew, J. E.; Law, J. T. *Bioconjugate Chem.* **2004**, *15*, 137.
(547) Weber, S. G.; Purdy, W. C. *Anal. Lett.* **1979**, *12*, 1.
(548) Di Gleria, K.; Hill, H. A. O.; McNeil, C. J.; Green, M. J. *Anal. Chem.* **1986**, *58*, 1203.
(549) Foulds, N. C.; Frew, J. E.; Green, M. J. In *Biosensors. A practical approach*; Cass, A. E. G., Ed.; IRL Press: Oxford, U.K., 1990; p 97.

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