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Oxidative phenol coupling—tyrosine dimers and libraries containing tyrosyl peptide dimers

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Abstract—The principles of phenol oxidation are outlined and summarized. Various procedures were used to dimerize tyrosine derivatives, especially linear tripeptides and cyclohexapeptides, via cross-linking of their phenolic side-chains. The coupling was performed by potassium hexacyanoferrate(III), phenyliodine(III)-bis(trifluoroacetate), vanadium(V)-oxyfluoride or horseradish peroxidase. The individual dityrosine and isodityrosine derivatives obtained in preparative scale, as well as the peptide-dimer libraries generated, were characterized by HPLC and electrospray ionization mass spectrometry. Further analyses were carried out by NMR spectroscopy, fluorescence spectroscopy and gas chromatography.

1. Introduction

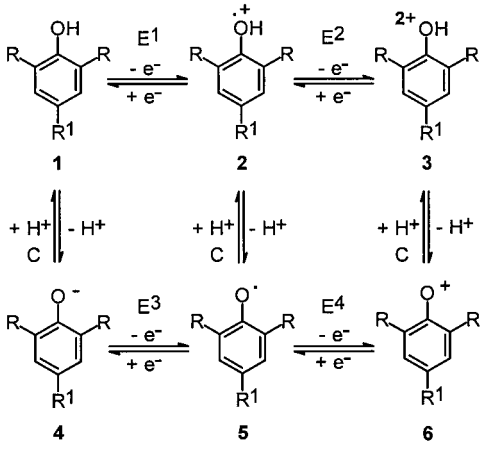
1.1. General aspects of the oxidative functionalization of phenols

A wealth of investigations has revealed that phenols are easily oxidized, both in vivo and in vitro, to give quinolide systems, which may be rearomatized or transformed into many other useful classes of compounds.

The primary oxidation process may be described by a 'scheme of squares', in which the individual species are correlated by *electron transfers* ('*E*' processes) and *proton transfers* ('C', chemical processes) (Scheme 1).[1,2]

Neutral phenol **1**, *cation radical* **2**, and *dication* **3** are linked by two successive one-electron transfers. All three species **1**–**3** are in equilibrium with their deprotonated forms, *anion* **4**, *neutral phenoxyl radical* **5**, and *phenoxenium cation* **6**, respectively, which, for their part, are again interrelated by two consecutive one-electron transfers. The real system is more complicated, since every species may react with each of the others via *bimolecular electron* or *proton transfers* to give two other species of the system. Moreover, other side reactions, such as dimerization of the radicals or electrophilic attack of the cations on nucleophiles, may take place.

Electroanalytical investigations[3–9] disclosed two important results, provided that dimerization of the radicals **2** and **5** is



Scheme 1. Square scheme of phenol oxidation; only one resonance structure of the species **1**–**6** is shown.

prohibited by means of steric hindrance and that strong nucleophiles are absent (for the case of sterically less hindered phenols in water or protic solvents see Refs. 10–12):

(i) In the *absence of a base*, phenol **1** itself is oxidized at E^1 (ca. 1000–1400 mV[‡]) to give the cation-radical **2** in a one-electron transfer. This species deprotonates to the neutral phenoxyl **5**, which is further oxidized at the applied potential to the desired cation **6**, because $E^4 < E^1$ (ECE reaction). Alternatively, **2** and **5** may react in a homogeneous redox reaction to produce **1** and again **6**.

(ii) In *the presence of a base*, the phenolate **4** formed from **1**

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[‡] In the following, all potentials refer to Ag/0.01 M Ag^+.

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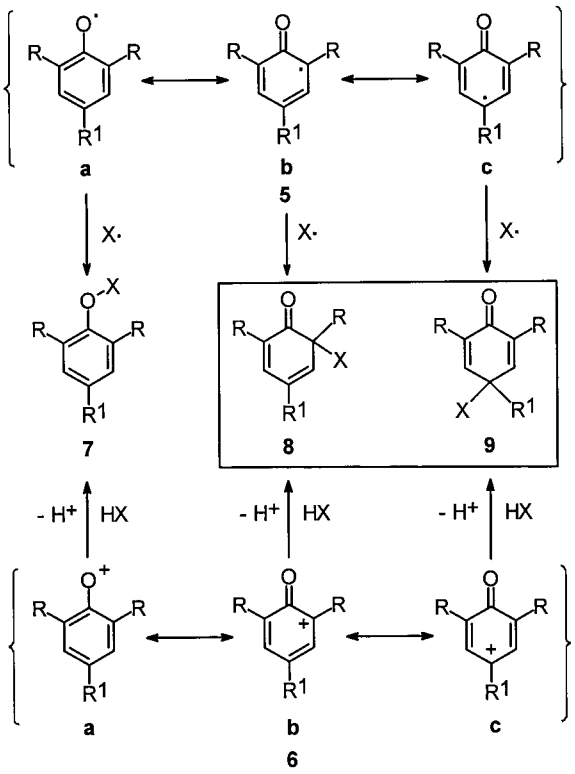


Scheme 2. Reaction paths of phenoxyls **5** and phenoxenium ions **6**.

is anodically oxidized to give the phenoxyl radical **5** at E^3=−600–+300 mV selectively (CE process). In special cases, the presence of **5** can be proved by electron spin resonance (ESR)[13] or multiple internal reflection Fourier transform infrared spectroscopy (MIRFTIRS).[14] At a higher potential, E^4=+700–800 mV, radical **5** is further oxidized in a one-electron transfer to the phenoxenium ion **6** (CEE mechanism), although **6** is not generally stable towards base.

Thus, phenoxyl **5** or phenoxenium ion **6** can be *selectively* generated using the proper potentials and proton concentration.

Scheme 1 is also valid for *chemical oxidants*. However, oxidant and substrate now have to come into contact in a bimolecular step influencing the kinetics. Moreover, the potential of the oxidant is fixed and cannot be changed at will as can that of an electrode; in some cases, a limited influence is possible via pH adjustment.[15,16] One also has to consider reactions of the oxidant with any of the species **1**–**6**, occurring as non-redox processes.

Potassium hexacyanoferrate(III), $K_3Fe(CN)_6$, in alkaline solution[15,17,18] is generally considered a genuine *one-electron* oxidant (oxidizing **4** to **5**). Other examples are metal complexes used in catalyzed oxygenations[19–23]with O_2 or alkylhydroperoxides, as well as PbO_2, Ag_2O, NiO_2, or oxygen radicals ($HO^{\cdot}$, *t*-$BuO^{\cdot}$, $ArO^{\cdot}$).[18] Apparently, also enzymes such as tyrosinase[24] and horseradish peroxidase (HRP)/H_2O_2[25–27] belong to this class of oxidants.

As *two-electron oxidants*, Tl^{3+}[28–31] or trivalent iodine compounds (for a recent literature survey see ref.[32]) such as iodobenzene diacetate (PIDA)[33–37] or iodobenzene bis(trifluoroacetate) (PIFA)[38–41] are often used. In the latter cases, a complex of the phenol **1** with the periodo compounds is formed,[33,34,38,41] the heterolysis of which produces an incipient phenoxenium ion **6** (formal two-electron oxidation). Similar reaction pathways are postulated for VOX_3[42] as oxidant although for vanadium the oxidation mechanism is rather complex. For Ce^{4+} mediated phenolic oxidations, the mechanism is equivocal[43] (for a more detailed account on phenol oxidants see Ref. 44).

The formation of the final reaction products will be controlled by the reactivity of the phenoxyls **5** and phenoxenium ions **6** (Scheme 2). Both species are stabilized by resonance (canonical structures **a**, **b**, **c**). In the case of the phenoxyls **5**, this can be proven by ESR measurements.[17,18,45] If the substituents R and/or R^1 show π-conjugation with the aromatic ring, further resonance structures with a free spin in these substituents also must be considered.[45]

Phenoxyls **5** can combine with other radicals $X^{\cdot}$, either added or produced from H–X via dehydrogenation by an excess of **5** (Eq. (1)):

$$H\text{–}X + \mathbf{5} \rightarrow H\text{–}X^{+\cdot} + \mathbf{4} \rightarrow X^{\cdot} + \mathbf{1} \quad (1)$$

$X^{\cdot}$ may be halogen atoms,[46] oxygen radicals (e.g. $^{\cdot}OOtBu$,[21,23] $^{\cdot}O\text{–}O^{\cdot}$,[46]. $^{\cdot}O\text{–}N{=}CR_2$[47]), nitrogen radicals (e.g. $^{\cdot}NO$,[48] $^{\cdot}NO_2$,[49] $^{\cdot}NHR$[50]), or carbon radicals (e.g. $^{\cdot}CH_2C_6H_5$[51]). Generally, the cyclohexadienones **8** and/or **9** are formed, while the ethers **7** are only observed with carbon radicals.[51]

Products **8** and **9** can also be formed from *phenoxenium* ions **6**(**a**,**b**,**c**) by addition of nucleophiles and deprotonation. Examples are the addition of H–X = HOH,[3,40,52–54] ROH,[31,33,35,37,52–54] ROOH,[52,53] RCOOH,[42,52–55] carbohydrates[2], RNH_2,[33,52,53,56] amides,[52,56] imines,[56] oximes,[56] amino acids (C- and N-terminal or via the side chain; useful for peptide syntheses).[2,57–60] Certain aromatic[61] and olefinic[39] nucleophiles react in a similar way.

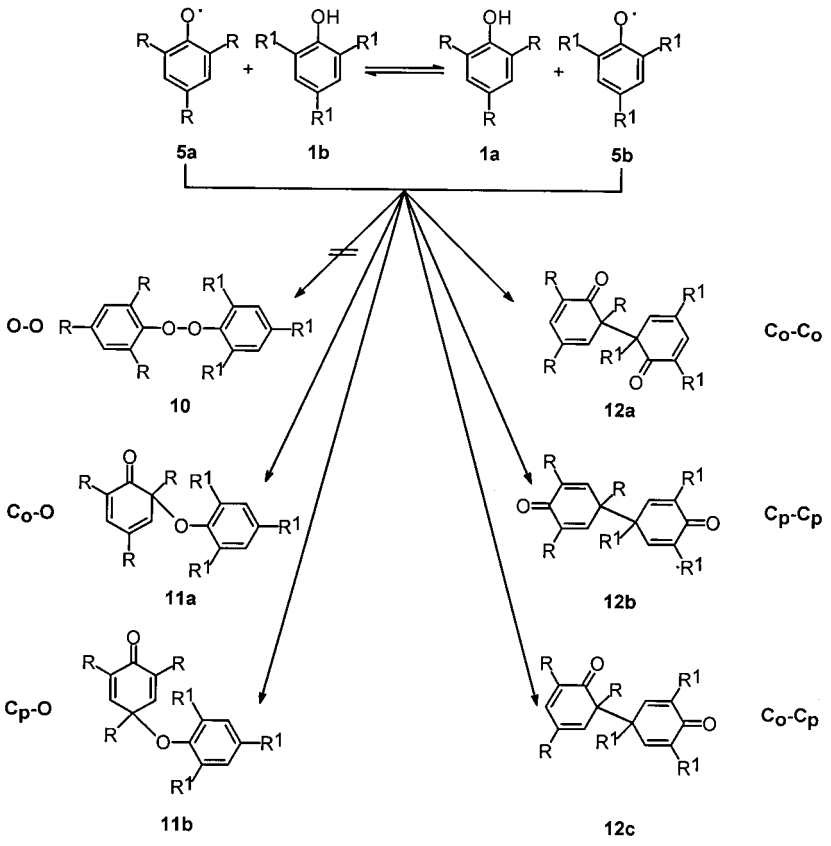
A certain selectivity in the formation of *ortho*- and *para*-quinolide structures **8** and **9** is ensured by the steric hindrance of R, R^1 and X, and by the electronic nature of R^1. Thus, if R^1 is electron-donating, **9** is favored; if R^1 is electron-withdrawing, **8** predominates.[51,62]

If phenol and nucleophile are combined in the same molecule via a spacer, generally ring closure to spirocyclohexadienones occurs upon oxidation.[30,33,36,52,63,64] This can be used for the synthesis of natural products such as alkaloids[41] or sponge metabolites.[29]

Moreover, cyclohexadienones **8** and **9** are synthetic equivalents used for the preparation of phenols, quinones, cyclohexadiene diols, quinol epoxides or cyclopentadienones, to mention a few applications.[65]

1.2. Oxidative phenol coupling

If $X^{\cdot}$ in Scheme 2 is another phenoxyl **5**, the resulting



Scheme 3. Coupling pattern of two phenoxyl radicals.

cyclohexadienones **8** and **9** are phenoxyl dimers. The principles of this dimerization are shown in Scheme 3.

Two phenoxyls **5a** and **5b** combine according to their free spin density distribution via O, C_o and C_p, thus leading to the coupling pattern shown: O–O (**10**), C–O (**11a**,**b**) and C–C (**12a**,**b**,**c**). If the phenoxyls **5** are identical (R=R^1), the process is denoted as 'homo-coupling'; for non-identical phenoxyls (R unequal R^1) the term 'hetero-coupling' is used. It should be mentioned that it is not necessary to prepare **5a** and **5b** separately. Due to electron and proton exchange between phenols and phenoxyls according to Scheme 3, we can start with phenol **1a**/phenoxyl **5b**, with phenol **1b**/phenoxyl **5a**, or even co-oxidize phenols **1a** and **1b**. With the exception of the aromatic peroxides **10**, all coupled species **11** and **12** have been found, [1,8,15,26,41,44,46,51,52,62,66–73] the type of coupling being dependent on the steric and electronic effects of the substituents R and R^1. [51,62,70a,74] Some rough rules can be derived:

(i) *homo-coupling:* R_{ortho} large, R_{para} small→**12b**
first R_{ortho} small, second R_{ortho} large, R_{para} large→**12a**
other cases→**11a**,**11b**,**12c**
(ii) *hetero-coupling:* R_{ortho} large, R_{para} any size, both electron-donating; R^1_{ortho} small, R^1_{para} any size →**11b**
R_{ortho} large, R_{para} any size, electron-withdrawing; R^1_{ortho} small, R^1_{para} any size and electronic effect →**11a**.

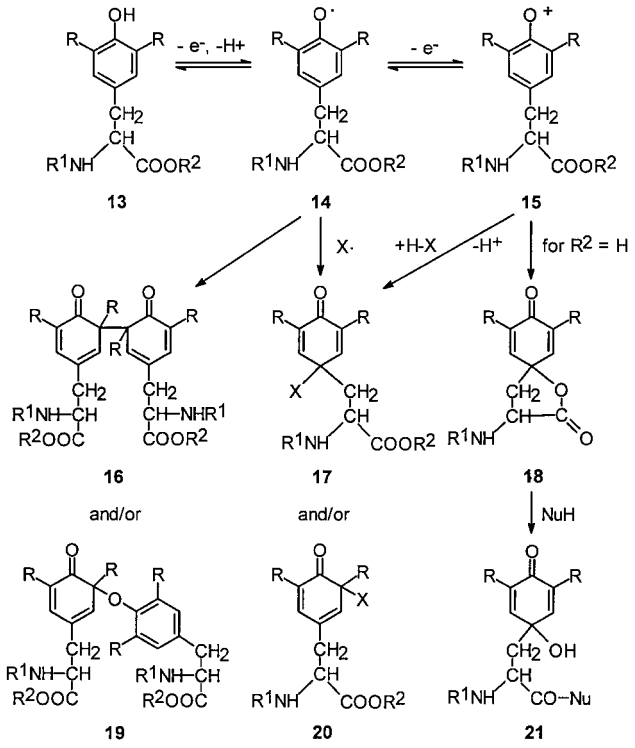
It should be emphasized that the coupling products **11** and **12** could, in principle, also result from the combination of phenoxenium ion **6** and phenol **1** with deprotonation (according to the lower part of Scheme 2). This may occur if the phenoxenium ion is formed incipiently (e.g. attack of the phenol on the complex formed from phenol/oxidant with simultaneous heterolysis at the complex center; formal two-electron oxidants, vide infra). On the other hand, a free phenoxenium **6** and phenol **1** would comproportionate into cation radical **2** and phenoxyl **5**, which would react according to Scheme 3. It is questionable whether selectivity with respect to CC or CO coupling can be obtained by selecting formal one- or two-electron oxidants.[15,26,43]

Additional diversity in coupling options appears if substituents R, R^1 are π-conjugated (coupling via an atom of the substituent[72a,75,76]) or for R, R^1=H (dienone→phenol rearrangement and oligomerization[43,76a,77]).

A very important case is the oxidation of *biphenols*, leading to *intramolecular* coupling of the resulting biradical. The ring closure again follows the principles of Scheme 3, the coupling pattern (C–C, C–O) being determined mainly by the molecular frame. A series of natural products and model compounds can be synthesized,[32,34,78–80] and also some natural products might in vivo be formed in this way,[81a] e.g. isoquinoline alkaloids,[44] ellagitannins,[81b] or lignin.[25,76,82]

1.3. Oxidation of tyrosine derivatives

Tyrosine, the only phenol among the essential amino acids,

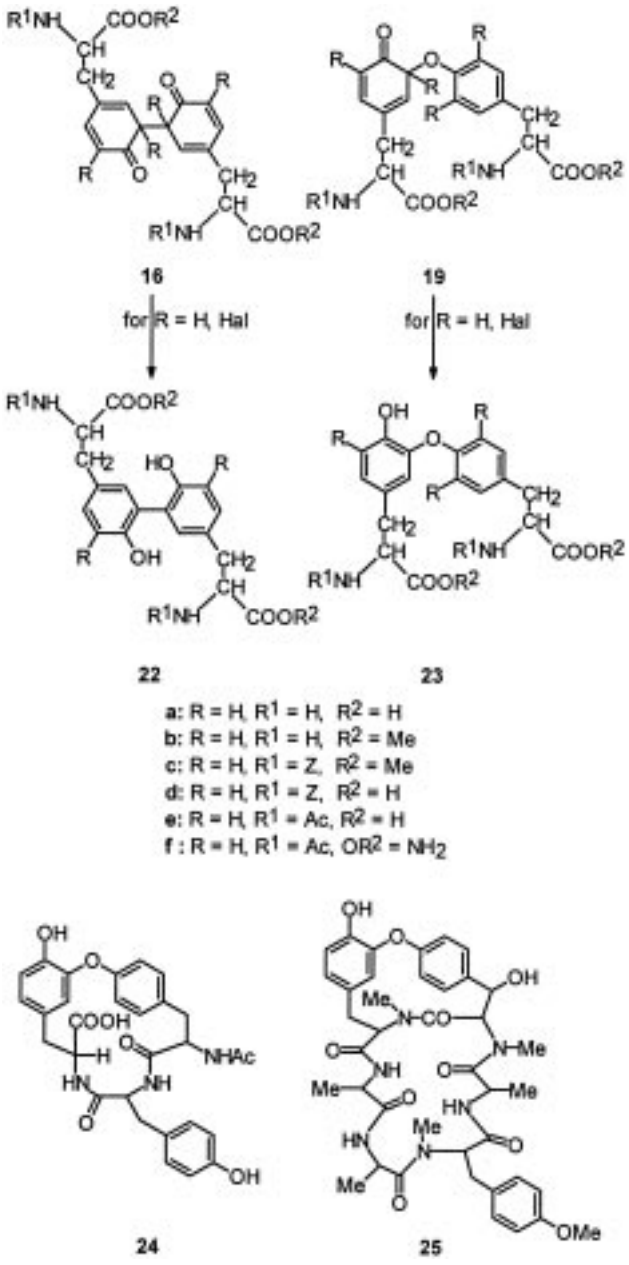


Scheme 4. Oxidative functionalization pathways of tyrosine derivatives.

and its derivatives **13** should undergo all the oxidative functionalization reactions discussed in the previous sections. One-electron oxidation to the phenoxyl **14** (Scheme 4), and dimerization of this species would lead to **16** or **19** (only C_o–C_o and C_o–O coupling is shown for simplicity), whereas reaction with other radicals X· would give the cyclohexadienones **17** and **20**. These species could also arise from the two-electron oxidation product **15** and HX, with intramolecular COOH addition (for R^2=H) to give spiroquinol ethers **18** as a special case. A heterolytic cleavage of **18** by nucleophiles NuH to the quinol **21** might also be envisaged.

Indeed, many of these reactions have been reported and were used as crucial steps to synthesize a series of natural and non-natural products. The electron/proton transfer equilibria **13**⇌**14**⇌**15** (see also Scheme 1) were characterized by cyclic voltammetry (CV).[67] Tyrosinoxyl radicals **14** were identified by ESR (organic solvents,[67a] enzymes[83]), and their conformational behavior was determined. They react with radicals such as *t*-BuOO· to give **17** (X= *t*-BuOO).[84] Reactions via phenoxenium ions **15** to give **17**, spirolactones **18** or the corresponding spirolactams are known (for earlier work of Wipf, Sharma, McKillop, Hara, Witkop see Ref. 67).[67,84,85a,b] Spirolactones **18** react with nucleophiles like C-terminal-protected amino acids to give (di)peptides **21** (Nu=amino acid residue) containing the tyrosine unit with a masked side chain,[67] which can be reduced to the phenolic structure.[67]

The dimerization of phenoxyls **14** is of high practical importance because the dimers **16** and **19** may rearrange or fragment to the *dityrosine* (**22**) or *isodityrosine* (**23**) systems (Scheme 5), especially if R=H or halogen (for hetero-



Scheme 5. Dityrosine and isodityrosine derivatives.

coupling of **14** with other phenoxyls according to Scheme 2).[67a,85c]

Namely, dityrosine **22a** and isodityrosine **23a** are structural elements in various peptidic natural products that may exhibit high biological activity such as the bastadines,[86] the enzyme inhibitor K-13 (**24**),[87] bouvardin (**25**),[88] vancomycin,[89] ristocetin,[90] or the antitumor agent RA-VII,[91] among others.[92] Dityrosine occurs as well in cell wall structural proteins, where the cross-links are responsible for stability due to restricted conformational flexibility (e.g. in yeasts[93]). It is also produced by irradiation of proteins and can be regarded as a marker of oxidative stress.[94]

Most of the above systems are cyclopeptides with **22a** or **23a** as ring constituents. Many different approaches through multi-step syntheses have been explored to achieve ring

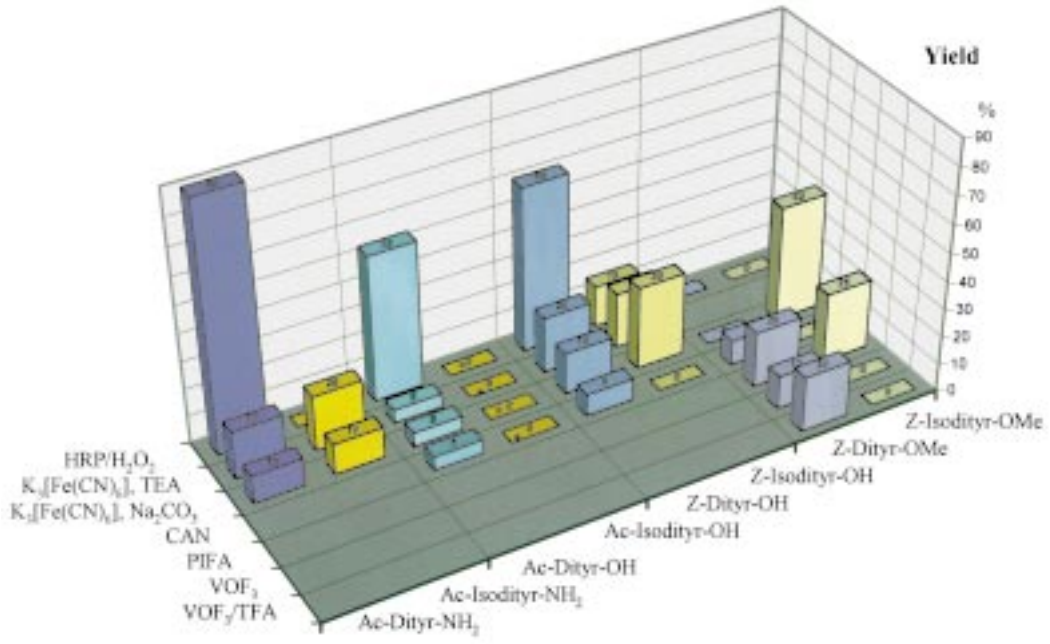


Figure 1. Comparison of oxidizing agents in the formation of tyrosine dimers **22** and **23**. Substrate oxidized: 30–32 μmol. Solvents: K_3FeCN_6/TEA, water/MeOH=1:1; $K_3Fe(CN)_6/Na_2CO_3$, water, and CH_2Cl_2/water/Bu_4NBr for Z-Tyr-OMe; CAN, water/MeOH, and MeOH for Z-Tyr-OMe; PIFA, CH_2Cl_2; VOF_3, CH_2Cl_2; VOF_3/TFA, CH_2Cl_2. *: Amount not determined.

closure by coupling of two tyrosines in a peptide chain via non-oxidative methods[89–91] (for a non-oxidative synthesis of **23a** itself see Ref. 95). Oxidative coupling (anode,[96] Tl^{3+},[79,97] enzymes[98]) of ring mono- or di-halogenated tyrosines was also successful and affords linear or cyclic systems containing **22a** or **23a**, whereas the direct chemical oxidative coupling of unsubstituted tyrosine has not been the subject of many publications[99] and was mainly achieved enzymatically.[99a–d]

It was our aim to develop on a preparative scale oxidative coupling procedures of peptides and cyclopeptides containing one tyrosine with an unsubstituted side chain. This would lead to cross linked peptides in one step, species which are of importance because the biphenyl or biphenyl ether cross-link will not be cleaved by proteases. Furthermore, receptor selectivity is usually better achieved by conformationally restricted cyclic ligands.

2. Results and discussion

According to the principles outlined in the previous chapters, oxidative coupling of tyrosines **13** should best proceed via the phenoxyl state **14**. Therefore, at first glance, anodic oxidation at constant potential in the presence of a base to ensure one-electron oxidation to **14** would appear as the method of choice. However, oligomerization or polymerization is expected to a great extent, if the phenol unit is unhindered in the *ortho*-positions, a well-known phenomenon in phenolic oxidations.[43,76a,77] Indeed, the electrode is rapidly coated, and the yield of dimers is very low.[84] Therefore, we first systematically investigated a variety of other oxidants, already characterized above [HRP/O_2, $K_3Fe(CN)_6$, $(NH_4)_2Ce(NO_3)_6$ (CAN), PIFA, VOF_3], for their ability to produce tyrosine coupling products **22** and/or **23** from the corresponding tyrosines **13** on a preparative scale. Some of the results obtained on an analytical scale are presented in Fig. 1.

Potassium hexacyanoferrate(III) in alkaline aqueous solutions led to rapid oligomerization and polymerization, whereas **13c** in a two-phase system, $CH_2Cl_2/Na_2CO_3/(Bu)_4NBr$, gave a good yield of **23c**. Application of triethylamine instead of inorganic bases in aqueous methanol produced mainly dityrosine, isodityrosine and trityrosine, and only small amounts of polymers from **13d**.

For the oxidative coupling of tyrosine derivatives (e.g. Z-Tyr-OMe **13c**, Fmoc-Tyr-OMe) which are poorly soluble or insoluble in aqueous solutions, *PIFA* in dichloromethane proved to be a suitable oxidative agent. The coupling proceeded under mild conditions and made accessible compounds **22c** and **23c** in preparative scale after purification by column chromatography on silica. The ratio of **22c** and **23c** could be influenced by variation of the amount of PIFA. The highest yield of the dityrosine was achieved with 2 mol equiv. of PIFA (36% **22c**, <5% **23c**). A lower yield of the dityrosine but the best yield of the isodityrosine was produced with 0.5 mol equiv. of PIFA (20% **22c,** 25% **23c**). In contrast to PIFA, VOF_3 (in acidic CH_2Cl_2) gave only dityrosine derivative **22c**. The oxidation with CAN in methanol/water or methanol was unsatisfactory in all cases.

Horseradish peroxidase (HRP) has been applied for catalytic oxidations of tyrosine derivatives with some success,[99a–d] as already mentioned. In most of our own experiments using a variety of substrates and HRP/H_2O_2 (the quality of the peroxidase is a crucial point), tyrosines were coupled selectively to dityrosine, but with **13d** only

Table 1. HRP catalyzed dimerization of tyrosine derivatives **13** (37°C, 1 h)

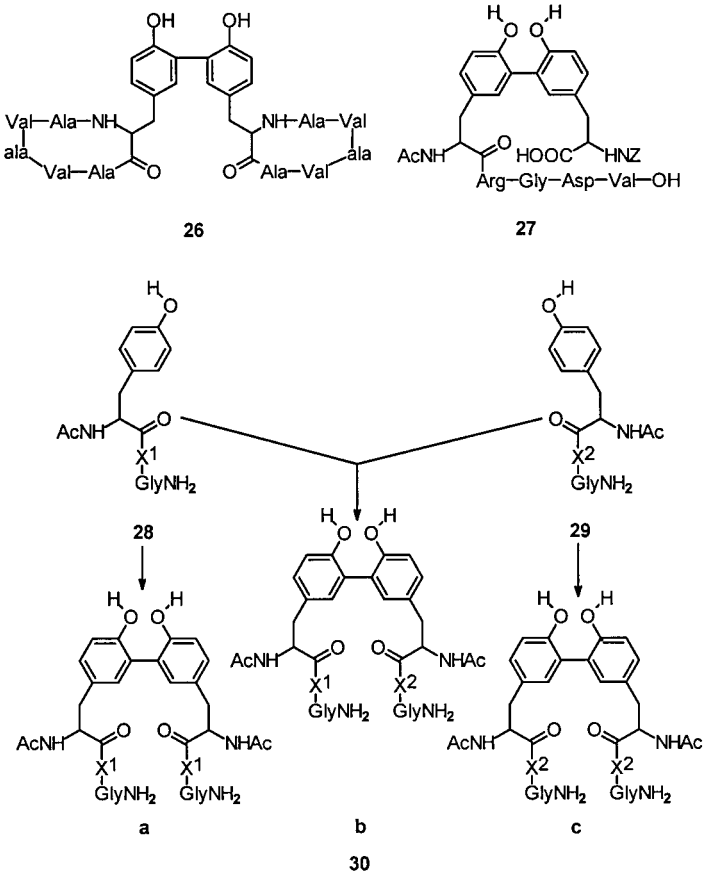
Solvent	Substrate	Yield of dityrosine (%)
0.2 M borate buffer	Ac-Tyr-NH_2	>90
0.2 M borate buffer	Z-Tyr-OH	60
0.2 M borate buffer	Ac-Tyr-OH	53
0.2 M borate buffer/20% MeOH	Ac-Tyr-NH_2	84
0.2 M borate buffer/33% MeOH	Ac-Tyr-NH_2	69
0.2 M borate buffer/50% MeOH	Ac-Tyr-NH_2	59
0.2 M borate buffer/66% MeOH	Ac-Tyr-NH_2	30
0.2 M borate buffer/50% dioxane	Ac-Tyr-NH_2	38
0.2 M borate buffer/50% *t*BuOH[a]	Ac-Tyr-NH_2	61
PBS buffer	Ac-Tyr-NH_2	47

[a] *t*BuOH:H_2O=4:1.

60% Z-Dityr-OH **22d** and also 19% Z-Isodityr-OH **23d** were formed. The best yields of dimeric products were achieved in 0.2 M borate buffer (pH 9.4),[99d] whereas in water and phosphate buffers (pH 7.4) only small amounts of the desired products were formed. In cases of low solubility of the substrates in the aqueous reaction medium, up to 50% of methanol or dioxane had to be used, which led on the one hand to a decrease in the yield of dimers (Table 1), but on the other hand was essential to make the oxidative coupling possible. In most cases, hydrogen peroxide was used in only 1.1-fold excess to prevent the production of oxidized compounds other than tyrosine dimers. Higher amounts of hydrogen peroxide promoted oligomerization and formation of various by-products.

The reactions were carried out at 37°C, according to Amado et al.[99d] Tests at room temperature showed lower yields, particularly with poorly soluble peptide mixtures. The reaction time was optimized by fluorescence measurements;[99a] in contrast to isodityrosine, dityrosine emits intensive fluorescence. Additionally, the samples were analyzed by HPLC and electrospray mass spectrometry. However, a differentiation of the isobaric dityrosine and isodityrosine derivatives is not possible by routine electrospray ionization mass spectrometry. Therefore, gas chromatography (GC) was used to assure the exclusive presence of dityrosyl compounds. The dimeric tyrosine derivatives and peptides (vide supra) were hydrolyzed and the amino acids transformed into *N*-trifluoroacetyl methyl esters whose GC analysis permitted the comparison with standards of tyrosine dimers.

After the HRP catalyzed oxidative coupling of tyrosines **13** was optimized, it was also used to couple small tyrosine peptides. Thus, cyclohexapeptide c(VAYAVa) (a=D-alanine) could be oxidized to the dityrosine-bridged product **26**. The ^{1}H NMR spectrum of **26** exhibited an ABX pattern of signals in the aromatic region similar to that of the dityrosine derivatives **22b**,**c** although the chemical shifts of individual protons varied with the type of groups at the N and C terminus. The amino acid components were determined by gas chromatography after total hydrolysis, and the structure was finally confirmed by HSQC- and HMBC-NMR measurements. The dimerization yield of **26** was 6.5%. Similarly, c(VAYASA) and c(VAYALa) could be dimerized in 6 and 5% yield, respectively (Scheme 6).



Scheme 6. HRP/H_2O_2 Coupling of tyrosine peptides.

Mixtures of cyclohexapeptides were also enzymatically dimerized. To avoid problems with solubility, peptides containing Lys, Ser, Arg, Asp and Glu were used. The cyclopeptide educts were purified by HPLC prior to use since a test reaction with unpurified peptides gave poor yields. In the enzymatically oxidized mixture of the three cyclopeptides c(VAYAVa), c(VAYASA), c(VAYALa), all possible dimeric coupling products could be detected by electrospray mass spectrometry; a selective hetero-cross coupling was not attained.

To obtain a hetero-cross coupled product in satisfactory yield, the pentapeptide Ac-Tyr-Arg-Gly-Asp-Val-OH was oxidized in the presence of a large excess of Z-Tyr-OH. After reaction and acidification, the unreacted Z-Tyr-OH as well as its dimers and oligomers could be easily extracted with ethyl acetate, thus leaving the nonreacted pentapeptide educt and the product **27** in the aqueous phase.

Peptide libraries are valuable tools in the random and rational screening of small molecule candidates to find new active compounds.[100] With respect to therapeutical applications, side-chain cross-links are desirable, because these bonds cannot be cleaved by peptidases. Therefore, we tested HRP catalyzed coupling in a preliminary feasibility study to demonstrate, in principle, the transformation of monomeric to dimeric tyrosyl-peptide libraries. Peptides of the general type Ac-Tyr-X-Gly-NH_2 (**28**,**29**) were chosen because *N*-acetylated peptide amides proved to be ideal substrates for the dimerization reaction. *N*-Protecting groups other than acetyl often caused low solubility of the peptides and a carboxy group at the C-terminus gave rise to oligomers via over-oxidation. X stands for all proteinogenic amino acids, except for tyrosine and cysteine which would themselves dimerize. Other oxidizable amino acids like tryptophan, phenylalanine, histidine or arginine did not cause problems. Additionally, aminobutyric acid (Abu) and ornithine (Orn) were incorporated in the X-position.

To assure that all possible dimers **30** were produced in the oxidations, test-sublibraries have been generated from mixtures containing four peptides each. The oxidative couplings with HRP were performed in fifty-eight wells of a microtitre-plate. Each one of the sublibraries consisted of four different homodimers and twelve heterodimers; but because of the symmetrical dityrosine bridge, only six of the latter were different. The same calculation for the whole library (from twenty peptides) resulted in twenty homodimers and one hundred and ninety heterodimers, a total of two hundred and ten dimers all together.

Characterization of the libraries was achieved by HPLC coupled to electrospray mass spectrometry. It was not possible to distinguish every single dimer analytically, neither in the entire library nor in the sublibraries because some of the peaks in HPLC chromatograms overlapped. Therefore, the libraries in total were separated by HPLC from other components and during this process consecutively subjected to MS. In this way, the mass peaks of all theoretically possible dimers could be observed, whereby only the peptides containing the isobaric amino acids leucine and isoleucine had identical masses. Although the presence of diisotyrosines could not be ruled out definitely, it is unlikely on account of the analysis of single tyrosyl-peptide dimers obtained by non-combinatorial procedures (vide supra).

The most striking result of the experiments using HRP as oxidant is that dityrosine formation prevails with low-molecular tyrosine derivatives and apparently occurs exclusively with tyrosyl peptides. This C_o–C_o regioselectivity may be attributed to a complex association of these tyrosyl peptides with each other, or, more likely, with the peptidic part of HRP in a way to arrange the phenolic moieties side by side. Since the spacer linking peptide and phenol moiety of the substrate extends to just one CH_2 group, C_o–C_o coupling of the phenoxyls formed by oxidation via the activated H_2O_2 should be easier than C_o–O coupling. This explanation is supported by the observation by G. N. Lewis et al.[76b] of a regio- and stereo-specific coupling of lignin precursors in the presence of a high-molecular peptide. These observations could open a new strategy in phenol coupling.

3. Conclusion

In this report, the principal features of oxidation and oxidative coupling of phenols have been compiled in the context of intra- and intermolecular coupling of tyrosine derivatives to various natural products. Systematic variation of oxidants revealed that HRP/H_2O_2 is the best oxidant to give dityrosine derivatives. The enzymatic method was then applied to the coupling of small linear and cyclic peptides, and libraries of biphenyl-cross linked peptides were assembled. These represent novel building blocks which may be used after deprotection for elongation of peptides or for further reactions of the cross coupled products.

4. Experimental

4.1. Materials and equipment

Solvents and chemicals for solid phase peptide synthesis were of p. a. quality (Fluka and Merck). Solid phase peptide synthesis was performed on a multiple peptide synthesizer (SMPS 350, Zinsser Analytic; Syro software, MultiSynTech, Bochum) by Fmoc/*t*Bu strategy. Fmoc-protected amino acids: Novabiochem (Bad Soden, Germany) and Bachem Biochemica GmbH (Bubendorf, Switzerland). Peptide amides were generated on Polystyrene A RAM resin (200–400 mesh, capacity: 0.57 mmol/g): Rapp Polymers (Tübingen, Germany). Cyclopeptides were synthesized on 2-chlorotritylchloride resin preloaded with an Fmoc-protected amino acid (1% DVB, 200–400 mesh, capacity: 1.3 mmol/g): Senn Chemicals AG (Dielsdorf, Germany). Horseradish peroxidase (525 U/mg): Boehringer GmbH (Mannheim, Germany). Microtitre-plates with 1 mL PP-wells: Costar GmbH, Bodenheim (Germany).

4.2. Analytical methods

4.2.1. Fluorescence measurements. Samples (10 μL) were taken after 5, 10, 15, 30, 45 and 90 min, diluted with 0.2 M borate buffer (3 mL) in a quartz cuvette and measured with a Luminescence Spectrometer LS5OB (Perkin–Elmer); room

temperature; excitation 300–400 nm (5 nm slit); emission maximum 385 nm.

4.2.2. Gas chromatography. Samples for routine analysis were obtained by hydrolysis of the peptides in 6N HC1 at 110°C for 24 h. The hydrolyzate was dried in an air current, and acetyl chloride in methanol was added. After 15 min at 110°C, the solvent was removed and the methyl esters dissolved in trifluoroacetic anhydride, which was removed after 10 min in an air stream.

4.2.3. HPLC and TLC. Analytical HPLC was carried out on a Nucleosil 300 C-18 column (250×2 mm, particle size: 5 μm; Grom, Herrenberg, Germany) using a Beckman HPLC-system (System-Gold, Beckman, San Ramon, USA), 214 nm. The flow rate was 0.3 mL/min. Water (0.12% trifluoroacetic acid, TFA) and acetonitrile (0.08% TFA) (E. Merck, Darmstadt, Germany) were used as solvents and a gradient from 10 to 100% acetonitrile in 45 min was chosen. Semipreparative HPLC was performed on a Waters 600 system (Waters, Milford, USA) and a Nucleosil 100 C-18 column (250×8 mm, particle size: 7 μm, Grom, Herrenberg, Germany), the flow rate was 3.5 mL/min. The libraries were desalted on RP-18 columns from ICT GmbH (Bad Homburg, Germany). For TLC silica gel $60/F_{254}$ on aluminum foil (E. Merck, Darmstadt, Germany) was used (solvent system: 80% chloroform, 30% methanol, 5% water). Compounds were detected with UV-light (254 nm and 366 nm) and by spraying with TDM-reagent.

4.2.4. Mass spectrometry. Electrospray ionization mass spectrometry (ESI-MS) was performed on an API III TAGA 6000 E triple-quadrupole mass spectrometer, equipped with an IonSpray™ interface (Sciex, Thornhill, Canada).

4.2.5. Nuclear magnetic resonance. NMR-Measurements were performed on Bruker spectrometers AM 250, WM 400 and AMX 600.

4.3. Synthesis of the starting peptides

4.3.1. Multiple peptide synthesis. Parallel reactions were performed in 2 mL-syringes, which were placed in reaction blocks with 96 positions, which permitted the synthesis either of several different peptides or of higher amounts of one single peptide, using several positions for only one sequence. The peptide synthesis on the resin (unloaded Rink-amide resin for peptide amides, preloaded Wang-resin for peptides with free C-terminus) was carried out in DMF, using 1-hydroxy-1H-benzotriazole (HOBt) and diisopropylcarbodiimide (DIC) for activation and piperidine for the cleavage of Fmoc-protective groups.

4.3.2. *N*-Acetylation of peptides. The peptide-loaded resin in the syringes was suspended in a mixture of CH_2Cl_2 (0.7 mL), ethyldiisopropylamine (DIPEA) (200 μL; 152 mg; 1.2 mmol) and acetic anhydride (100 μL; 108 mg; 1.1 mmol). After 1 h at room temperature in a shaker, the resin was washed several times with CH_2Cl_2 and diethyl ether.

4.3.3. Cleavage of the peptide amides from the resin. The peptide-loaded resins were suspended in a mixture of 82.5% TFA, 5% water, 5% phenol, 5% thioanisole and 2.5% ethanedithiol (reagent K) (1 mL) and shaken for 3 h at room temperature. The cleavage solution was dropped in an ice-cold mixture of diethyl ether/*n*-hexane (1:1) to precipitate the peptides. The precipitates were centrifuged and washed three times with diethyl ether/*n*-hexane (1:1). The pellets were dissolved in *tert*-butylalcohol/water (4:1), deep-frozen in liquid nitrogen, lyophilized, and analyzed with ESI-MS and analytical HPLC and used without further purification.

4.3.4. Cyclopeptides. The corresponding linear hexapeptides (H-VAYAVa-OH, H-VAYALa-OH, H-VAYASA-OH) were synthesized on Fmoc-amino acid loaded 2-chlorotrityl-PS/1%DVB resin. The cleavage from the resin was achieved in a mixture of CH_2Cl_2 and 1,1,1,3,3,3-hexafluoroisopropanol (7:3) in 1 h. The cleavage solutions were evaporated and the side-chain protected peptides dissolved in DMF to form a 1 mmol solution. For cyclization 4 equiv. of DIPEA and 3 equiv. of a 0.4 mol solution of *O*-(benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate/HOBt were added dropwise. After 3–5 h (TLC-monitoring), the solvent was evaporated. The side-chain protected cyclopeptides were dissolved in CH_2Cl_2 and extracted with 5% $KHSO_4$, water, 5% $NaHCO_3$ and water (3 times each). The organic phase was concentrated in vacuo. Side-chain deprotection was performed in 95% TFA. After evaporation the products were lyophilized from *tert*-butylalcohol/water (4:1), purified by semipreparative HPLC (214 nm) and characterized by analytical HPLC and ESI-MS.

4.4. Coupling with chemical oxidants

4.4.1. Oxidation of Z-Tyr-OH with $K_3Fe(CN)_6$/TEA. Z-Tyr-OH (100 mg, 0.32 mmol) in a mixture of MeOH (2 mL), water (3 mL) and triethylamine (TEA) (0.73 g, 7.2 mmol) was stirred for 1 h under Ar. $K_3Fe(CN)_6$ (200 mg, 0.61 mmol) was added and stirring continued for 12 h. The reaction was quenched by adding 5% aqueous $KHSO_4$, the solvents were evaporated in vacuo and the residue exhaustively extracted with MeOH. The combined methanolic fractions were concentrated to 5 mL. Preparative HPLC (40→65% MeCN in 30 min) gave 19 mg (30 μmol, 19%) Z-Dityr-OH **22d**, 21 mg (34 μmol, 21 %) Z-Isodityr-OH **23d**, and 12 mg (14 μmol, 13%) Z-Trityr-OH.

22d. ESI-MS [amu]: 629.5 $[M+H]^+$. ^{13}C NMR (100 MHz, CD_3OD): δ 39.3, 58.2, 68.9, 118.8, 128.7, 129.9, 130.2, 130.7, 131.2, 131.5, 131.9, 150.0, 139.5, 155.3, 159.7, 175.4.

23d. ESI-MS [amu]: 629.5 $[M+H]^+$. ^{13}C NMR (100 MHz, CD_3OD): δ 38.2, 39.4, 57.9, 58.1, 67.5, 116.8, 118.0, 128.3–134.6, 139.0, 139.8, 154.2, 155.1, 159.8, 160.2, 177.0, 178.3.

Z-Trityr-OH. ESI-MS [amu]: 942.5 $[M+H]^+$. ^1H NMR (100 MHz, CD_3OD): δ 3.02 (3H, m), 3.23 (3H, m), 4.50 (3H, m), 5.08 (3H, d, J=12.9 Hz), 5.12 (3H, d, J=11.9 Hz),

6.92 (2H, d, J=8.2 Hz), 7.14 (2H, d, J=7.9 Hz), 7.21 (4H, m), 7.34 (15H, s).

4.4.2. Oxidation of Z-Tyr-OMe with phenyliodine(III) bis(trifluoroacetate). A solution of PIFA (1.29 g, 3.0 mmol) in CH_2Cl_2 (9 mL) was added dropwise to a solution of Z-Tyr-OMe (1.0 g; 3.0 mmol) in dry CH_2Cl_2 (45 mL). After 1.5 h, $NaHCO_3$ (504 mg, 6.0 mmol) was added, and the mixture stirred for 5 min. After filtration through celite and concentration in a rotary evaporator, the remaining syrupy raw product was chromatographed on silica gel (eluent: petroleum ether/ethyl acetate 2:1). Two fractions were collected: Z-Isodityr-OMe **23c** (R_f=0.36, petroleum ether/ethyl acetate 1:1), 106 mg (162 μmol; 10.6%), ESI-MS [amu]: 657.5 $[M+H]^+$, and Z-Dityr-OMe **22c** (R_f=0.25, petroleum ether/ethyl acetate 1:1), 219 mg (334 μmol; 22%), ESI-MS [amu]: 675.5 $[M+H]^+$. Both species were characterized by ^1H- and ^{13}C NMR (not shown).

In order to simplify the NMR spectra, the Z protecting groups of **22c** and **23c** were cleaved with ammonium formiate and palladium black.[101] Semipreparative HPLC (10⟶40% acetonitrile/water, 30 min) yielded 22 mg (57 μmol; 75%) of H-Dityr-OMe **22b** and 18 mg (46 μmol; 61%) of H-Isodityr-OMe **23b**.

22b. ESI-MS [amu]: 389.5 $[M+H]^+$. ^1H NMR (250 MHz, CD_3OD): δ 3.10 (1H, dd, J=7.6, 14.5 Hz), 3.23 (1H, dd, J=5.7, 14.4 Hz), 3.81(6H, s), 4.28 (2H, dd, J=5.7, 7.6 Hz), 6.92 (2H, d, J=7.6 Hz), 7.07 (2H, s), 7.10 (2H, d, J=7.6 Hz). ^{13}C NMR (63 MHz, CD_3OD): δ 37.7, 54.7, 56.5, 118.7, 127.4, 128.5, 132.0, 134.6, 156.4, 171.6.

23b. ESI-MS [amu]: 389.5 $[M+H]^+$. ^1H NMR (400 MHz, CD_3OD): δ 2.92 (4H, m), 3.53 (3H, s), 3.59 (3H, s), 4.05 (2H, m), 6.63 (1H, s), 6.70 (2H, d, J=8.6 Hz), 6.74 (2H, s), 6.98 (2H, d J=8.6 Hz). ^{13}C NMR (400 MHz, CD_3OD): δ 36.7, 53.5, 55.3, 118.3, 118.8, 123.5, 126.8, 127.5, 129.1, 131.6, 144.7, 150.3, 159.2, 170.4.

4.5. Enzymatic oxidative coupling

4.5.1. Enzymatic oxidative coupling of a cyclopeptide. The cyclopeptide c[VAYAVa] (46 mg; 80 μmol) was dissolved in 0.2 M borate buffer (45 mL) and HRP (2.5 mg; 1340 U) and 3% hydrogen peroxide (650 μL; 580 μmol) were added. After 12 h at 37°C the reaction mixture was acidified with dilute HCl and the solvent removed in a rotary evaporator. Semipreparative HPLC yielded 3 mg (2.6 μmol; 6.5%) of dimeric cyclopeptide **26**.

ESI-MS [amu]: 1147.5 $[M+H]^+$, 1169.5 $[M+Na]^+$. ^1H NMR (600 MHz, DMSO-d_6): δ 0.85 (24H, m), 1.18 (6H, d, J=6.9 Hz), 1.28 (12H, m), 2.00–2.30 (4H, m), 3.00 (4H, d, J=7.4 Hz), 3.89–4.37 (12H, m), 6.78 (2H, d, J=8.3 Hz), 6.95 (2H, dd, J=2.2, 8.3 Hz), 6.97 (1H, d, J=2.2 Hz), 7.19 (2H, J=7.6 Hz), 7.84 (2H, d, J=7.4 Hz), 8.03 (6H, m), 8.42 (2H, d, J=5.5 Hz), 9.04 (2H, s).

4.5.2. Enzymatic cross-coupling. Ac-Tyr-Arg-Gly-Asp-Val-OH (20 mg; 31 μmol) and Z-Tyr-OH (32 mg; 100 μmol) were dissolved in 0.2 M borate buffer (3 mL). HRP (1.4 mg; 750 U) in buffer (750 μL) and 3% H_2O_2 (90 mL) were added. The mixture was maintained at 37°C for 45 min, after acidification diluted with water and extracted 5 times with ethyl acetate (50 mL). The aqueous phase was desalted and analyzed by HPLC and ESI-MS. Semipreparative HPLC: 7.7 mg of cross-coupled **27** (8 μmol; 26%).

ESI-MS [amu]: 964.5 $[M+H]^+$. ^1H NMR (400 MHz, CD_3OD): δ 0.94 (3H, d, J=6.8 Hz), 0.95 (3H, d, J=6.8 Hz), 1.57–1.73 (2H, m), 1.95 (3H, s), 1.82–1.98 (2H, m), 2.13–2.22 (1H, m), 2.69–3.30 (7H, m), 2.74 (1H, d, J=7.5 Hz), 3.76 (1H, d, J=17 Hz), 3.82 (1H, d, J=17 Hz), 4.23–4.80 (4H, m), 4.50 (1H, t, J=7.6 Hz), 5.01 (1H, d, J=13 Hz), 5.05 (1H, d, J=13 Hz), 6.81 (1H, d, J=8.0 Hz), 6.84 (1H, d, J=8.0 Hz), 7.08 (2H, dd, J=3.3, 8.0 Hz), 7.13 (1H, d, J=3.3 Hz), 7.16 (1H, d, J=3.3 Hz), 7.26 (5H, m); only shifts of protons bound to carbons are given.

4.6. Tripeptide dimer libraries by enzymatic oxidative coupling

4.6.1. General. For optimization test reactions were performed in microtitre-plates with 250 μL-wells containing 5 μmol of the peptide mixture in 200 μL buffer. Horseradish peroxidase (0.1 mg; 50 U) in 30 μL solvent and 6 μL 3% hydrogen peroxide were added and the microtitre-plate incubated at 37°C for 1.5 h.

4.6.2. Synthesis of dimeric sublibraries in small scale. Tripeptide Ac-YXG-NH_2 mixtures (30 μmol) and 300 U (0.57 mg) horseradish peroxidase in 0.2 M borate buffer or borate buffer/dioxane (1:1) (950 μL) were placed in 1 mL wells of a microtitre-plate. After addition of 3% hydrogen peroxide (36 μL) the oxidations were performed at 37°C for 1.5 h. The mixtures were carefully acidified with 5% HCl and samples were taken for analytical characterization.

4.6.3. Synthesis of the complete tripeptide dimer library in preparative scale. HRP (6.5 mg; 3400 U) and 3% H_2O_2 (408 μL) were added to Ac-YXG-NH_2 (340 μmol; 17 μmol of each peptide) in 0.2 M borate buffer (11 mL). The solution was incubated for 1.5 h at 37°C. After careful acidification with 5% HCl, the work-up was performed as described below, and the dimeric peptide library was characterized by ESI-MS.

4.6.4. Purification methods. Horseradish peroxidase was removed from the samples by centrifugation in Amicon microconcentrators with a molecular weight cut-off of 3000 Da. The peptide libraries were desalted on RP-18 columns: The columns were equilibrated with 10% acetonitrile/water. The buffer solutions were placed on top of the column and diluted with water to wash off the salts. Then the peptides were eluted with 50% acetonitrile/water.

HPLC-purifications were performed on RP-18 columns in semipreparative scale using acetonitrile (0.1% TFA) and water (0.1% TFA) as solvents. The gradient was 10 to 50% acetonitrile in 45 min.

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