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A study to develop platinum(IV) complex chemistry for peptide disulfide bonds formation

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Abstract: Platinum(IV) complexes with a heterocyclic ligand and ancillary ligand have been investigated and applied in treating various tumour cell lines. Another application of the Pt(IV) complexes in forming peptide disulfide bonds was investigated in this work. For development of the Pt(IV) complex chemistry for disulfide bonds formation in peptides, two Pt(IV) complexes, $[PtCl_2(phen)(en)]Cl_2$ and $[PtCl_2(bpy)(en)]Cl_2$, were synthesized and characterized using elemental analysis, ESI-MS and NMR. Subsequently, they were investigated as the oxidants for the formation of disulfide bonds in various peptides. Excellent purities and yields of disulfide-containing peptides were achieved when the reactions were carried out in aqueous solution. The reactions were completed rapidly in a wide range of pH even in acidic medium at room temperature. Intramolecular-disulfide bond was formed in each of peptides in the solution containing two dithiol-containing peptides, making the Pt(IV) complexes are useful for disulfide-containing peptide libraries. Additionally, the two Pt(IV) complexes can be used as oxidants for the synthesis of disulfide bonds on resin, making the synthesis more automatically in manufacture.

Keywords: platinum(IV) complexes, peptide, disulfide bond, oxidation

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The platinum(IV) complexes with a heterocyclic ligand (1,10-phenanthroline (phen) or methyl substituted phen) and ancillary ligand (*S,S* or *R,R* isomer of 1,2-diaminocyclohexane or ethylenediamine) have raised interest due to their encouraging cytotoxicity results *in vitro*.[1] Pt(IV) complexes possess six-coordinate octahedral geometry and are kinetically inert. Their anticancer activities are triggered by a two-electron reduction.[1] The reductants involve cysteine, homocysteine, glutathione, ascorbic acid *et al*. Among these reductants, thiol-containing compounds can be oxidized to their disulfide forms.[1,2] Therefore, the anticancer active Pt(IV) complex may be used as an oxidant for synthesis of disulfide bonds in peptides. Recently, we synthesized a phen-liganded Pt(IV) complex *trans*-$[Pt(OH)_2(phen)(en)]^{2+}$, it was successfully used as an oxidant for formation of peptide disulfide bonds in a aqueous solution.[3] An additional two Pt(IV) complexes, *trans*-$[PtCl_2(CN)_4]^{2-}$ and *trans*-$[PtCl_2(en)_2]^{2+}$, have also been investigated for the purpose of forming peptide disulfides.[4] However, *trans*-$[PtCl_2(CN)_4]^{2-}$ can oxidize methionine residue to methionine sulfoxide, making it unsuitable for the formation of disulfide bond in a methionine-containing peptide. *trans*-$[PtCl_2(en)_2]^{2+}$ is a very efficient reagent in an optimal pH 4-5 buffer solution.[5] The reaction time is increased dramatically with the decreasing of pH.

Peptide-based drugs containing disulfide bonds have been successfully used for the treatment of various diseases. Moreover, a great many of peptide candidates are developed in clinical trials. Disulfide bond helps contribute to stabilizing a peptide's secondary structure, which can promote its activity, selectivity and proteolytic stability.[6] Hence, the construction of disulfide bonds is one of the critical steps in peptide synthesis. Generally, disulfide bonds formation can be achieved through oxidation of thiol moieties of two cysteine residues *via* homogeneous or

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heterogeneous reactions. The oxidants including iodine, oxygen, thallium(III) trifluoroacetate, carbon tetrachloride, dimethyl sulfoxide (DMSO), *trans*-3,4-dihydroxyselenolane oxide (DHS), *N*-chlorosuccinimide (NCS) and 3-nitro-2-pyridinesulfenates (NPys) have been used for the preparation of peptide disulfide bonds. However, Drawbacks such as low yield, long reaction time and the formation of side products are often observed in the oxidation reactions.[7] Therefore, the research and development of an efficient oxidant has grown steadily.

In order to develop the Pt(IV) complex chemistry for the preparation of disulfide bonds in peptides. 1,10-Phenanthroline (phen) and 2,2′-dipyridine (bpy) substituted one of the ethylenediamine ligands of *trans*-$[PtCl_2(en)_2]^{2+}$, affording two Pt(IV) complexes *trans*-$[PtCl_2(phen)(en)]^{2+}$ and *trans*-$[PtCl_2(bpy)(en)]^{2+}$. The two Pt(IV) complexes were synthesized according to our previous work.[1f,1g]

To investigate whether methionine can be oxidized to methionine sulfoxide by the Pt(IV) complex. The reaction between *trans*-$[PtCl_2(phen)(en)]^{2+}$ and methionine was firstly studied by NMR. As shown in Figure S1, the initial 2.07 and 2.58 ppm resonances of the CH_3 and CH_2 groups bonded to sulfur, respectively, were replaced by resonances at 2.68 and 3.10 ppm, respectively. These changes in the chemical shift are in the direction of formation of methionine sulfoxide, that is observed in the reaction of *trans*-$[PtCl_2(CN)_4]^{2-}$ with methionine (see Fig. S2).[8] However, no methionine sulfoxide was observed when peptide 1 (Sequence: CGYCHKLHQMK-NH_2) was reacted with *trans*-$[PtCl_2(phen)(en)]^{2+}$ and *trans*-$[PtCl_2(bpy)(en)]^{2+}$ (Fig. 1). Disulfide bond was formed immediately at room temperature. Excellent purity of disulfide-containing peptide 1 was achieved in aqueous solution. Moreover, no side reaction on tyrosine residue was observed, confirming that the Pt(IV) complexes can not oxidize the tyrosine residue in peptide.

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In order to investigate whether the two Pt(IV) complexes can oxidize tryptophan residue in peptide. Reduced somatostatin was reacted with the Pt(IV) complexes, no tryptophan related oxidation product was observed after the reaction.

The oxidation reactions between each of the Pt(IV) complexes and peptide 2 (Sequence: CGYCHKLHQGK-NH_2) were investigated in various solvents. The results are shown in Table 1. Excellent purities of oxidized peptide 2 are achieved in various solvents. Therefore, the Pt(IV) complexes tolerate a range of hydrogen ion concentration upon the formation of peptide disulfide bonds, and the reaction time increased with the increasing of the concentration of hydrogen ion.

Eight dithiol-containing peptides with variable lengths were selected for demonstrating the efficiency of the two Pt(IV) complexes for disulfide bonds formation; HPLC chromatograms and ESI-MS spectra are displayed in the Supporting Information. The results are summarized in Table 2. As can be seen, excellent purities and yields were obtained for all disulfide-containing peptides. By comparison, formation of disulfide bond in oxytocin *via* oxgen oxidation was also studied in this work. It needs about 22 h to convert dithiol group to disulfide bond with a yield of 97% in pH 7.95 buffer solution (Fig. S62). Formation of disulfide bond in oxytocin *via* the Pt(IV) complex oxidation was completed in less than one minute, while no disulfide bond was formed in 24 h *via* oxygen oxidation in 10.0 mM of HCl solution. Moreover, the oxidation products are easily separated by HPLC, and the Pt(IV) complexes and their corresponding reduction product, Pt(II) complexes, can be collected, regenerated and reused for the formation of disulfide bond.

To investigate the applicability of the Pt(IV) complex oxidation method with multiple-disulfide-containing peptide, reduced α-conotoxin SI (a 13-residue peptide from a piscivorous cone snail, and it contains two disulfide bonds and a C-terminal

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amide;[9] Sequence: ICCNPACGPKYSC-NH_2) was used as obtained and analyzed by HPLC (Figure 2a). The reaction between excess of *trans*-$[PtCl_2(phen)(en)]^{2+}$ and reduced α-conotoxin SI was investigated. As shown in Figure 2b, isomers (Cys^2-Cys^3/Cys^7-Cys^{13} and Cys^2-Cys^{13}/Cys^3-Cys^7) and α-conotoxin SI (Cys^2-Cys^7/Cys^7-Cys^{13}) were observed. The ratio of isomers to α-conotoxin SI is 47% : 53%, which was calculated by the peak area.

The reaction between *trans*-$[PtCl_2(phen)(en)]^{2+}$ and the mixture of two dithiol-containing peptides (peptide 1 and reduced phenypressin) was studied by HPLC (Fig. 3). The yields of peptide 1 and phenypressin are all almost 100%. No intermolecular disulfide bond was formed between two peptides. Therefore, the Pt(IV) complex is a very excellent oxidant for the synthesis of intra-molecular disulfide bonds in a solution containing two peptides. Hence, the Pt(IV) complex is useful for disulfide bond containing peptide libraries.

The applicability of the Pt(IV) complex oxidation method for on-resin disulfide bonds formation was investigated in this work. On-resin oxytocin (sequence: CYINQCPLG-NH_2), isotocin (sequence: CYISNCPIG-NH_2) and iRGD (sequence: CRGDKGPDC-NH_2) with S-Mmt-protected cysteine residues were used as obtained. The Mmt protected group was removed by CH_2Cl_2 solution with 1% TFA, the obtained peptidyl resin was reacted with *trans*-$[PtCl_2(phen)(en)]^{2+}$ or *trans*-$[PtCl_2(bpy)(en)]^{2+}$. After the reaction, oxytocin, isotocin and iRGD peptide cleaved from the resin were analyzed by HPLC (Fig. 4). As can be seen, peptide disulfide bonds were successfully formed on resin with the yields of 85%, 85% and 82% for oxytocin, isotocin and iRGD, respectively. Moreover, the reactions can be carried out automatically on a peptide synthesizer, making the peptide disulfide synthesis more easily to manufacture.

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Reduction of excess of Pt(IV) complex *trans*-$[PtCl_2(phen)(en)]^{2+}$ by a reduced iRGD peptide was studied by ^1H NMR. NMR spectrum is shown in Figure S3. As can be seen, *trans*-$[PtCl_2(phen)(en)]^{2+}$ was reduced to its correspondence Pt(II) complex with the loss of two axial chloride ligands. Moreover, conversion the dithiol group of reduced iRGD peptide to disulfide bond was best achieved when the mole ratio of Pt(IV) complex to reduced iRGD peptide was 1:1. The reaction mechanism for oxidative formation of disulfide bond by Pt(IV) complex has been studied, a mechanism named chloride-mediated reductive elimination reaction has been widely accepted as the main pathway for reduction of the Pt(IV) complex.[2] Based on our above results, the mechanism for the synthesis of disulfide bonds by *trans*-$[PtCl_2(phen)(en)]^{2+}$ and *trans*-$[PtCl_2(bpy)(en)]^{2+}$ was proposed as displayed in Scheme 1.

In summary, the Pt(IV) complexes, *trans*-$[PtCl_2(phen)(en)]^{2+}$ and *trans*-$[PtCl_2(bpy)(en)]^{2+}$, are efficient reagents for the formation of disulfide bonds in various solvents. Disulfide bonds formation can be achieved with excellent purities and yields at room temperature. Formation of peptide disulfides were successfully achieved in a solution containing two peptides, making the Pt(IV) complexes are useful for disulfide-containing peptide libraries. Additionally, the two Pt(IV) complexes can be used as excellent oxidants for the synthesis of disulfide bonds on resin, making the synthesis of peptide disulfides more automatically in manufacture.

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Conflicts of interest

There are no conflicts to declare.

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Table 1. Pt(IV) complexes reaction with peptide 2 in various solvents.

No.	Solvent	$[H^+]$/(mol/L)	Pt(IV) complex	Purity of oxidized peptide [a] (%)	Reaction time (min)
1	phosphate buffer	10^{-7}	*trans*-$[PtCl_2(phen)(en)]^{2+}$	97	< 1
			trans-$[PtCl_2(bpy)(en)]^{2+}$	97	< 1
2	acetate buffer	$10^{-4.2}$	*trans*-$[PtCl_2(phen)(en)]^{2+}$	96	< 1
			trans-$[PtCl_2(bpy)(en)]^{2+}$	97	< 1
3	HCl	0.1	*trans*-$[PtCl_2(phen)(en)]^{2+}$	96	< 1
			trans-$[PtCl_2(bpy)(en)]^{2+}$	97	< 1
4	HCl	0.5	*trans*-$[PtCl_2(phen)(en)]^{2+}$	95	～ 5
			trans-$[PtCl_2(bpy)(en)]^{2+}$	96	～ 5

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5	HCl	1.0	*trans*-$[PtCl_2(phen)(en)]^{2+}$	97	~ 20
			trans-$[PtCl_2(bpy)(en)]^{2+}$	96	~ 20
6	H_2O		*trans*-$[PtCl_2(phen)(en)]^{2+}$	97	< 1
			trans-$[PtCl_2(bpy)(en)]^{2+}$	96	< 1

[a] Purities of oxidized peptides were calculated by the areas of the chromatographic products peaks recorded at 215 nm; Peak areas of Pt(IV)/Pt(II) are not included when calculating the purities of oxidized peptides.

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Table 2. Synthesis of peptide disulfides by Pt(IV) complexes in 10.0 mM of HCl solution.

No.	Peptide	Sequence of peptide	Purity of reduced peptide [a] (%)	Pt(IV) complex	Purity of oxidized peptide [b] (%)	Reaction time (min)	Yield of oxidized peptide (%)
1	peptide 1	CGYCHKLHQMK-NH_2	99	*trans*-$[PtCl_2(phen)(en)]^{2+}$	99	< 1	99
				trans-$[PtCl_2(bpy)(en)]^{2+}$	99	< 1	99
2	peptide 2	CGYCHKLHQGK-NH_2	99	*trans*-$[PtCl_2(phen)(en)]^{2+}$	97	< 1	99
				trans-$[PtCl_2(bpy)(en)]^{2+}$	96	< 1	99
3	reduced oxytocin	CYINQCPLG-NH_2	75	*trans*-$[PtCl_2(phen)(en)]^{2+}$	80	< 1	96
				trans-$[PtCl_2(bpy)(en)]^{2+}$	77	< 1	96
4	reduced arginine vasopressin	CYFQNCPRG-NH_2	88	*trans*-$[PtCl_2(phen)(en)]^{2+}$	98	< 1	99
				trans-$[PtCl_2(bpy)(en)]^{2+}$	96	< 1	99
5	reduced somatostatin	AGCKNFFWKTFTSC-OH	90	*trans*-$[PtCl_2(phen)(en)]^{2+}$	90	< 1	97
				trans-$[PtCl_2(bpy)(en)]^{2+}$	90	< 1	97

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6	reduce brain binding peptide	CLSSRLDAC-NH_2	70	*trans*-$[PtCl_2(phen)(en)]^{2+}$	74	< 1	97
				trans-$[PtCl_2(bpy)(en)]^{2+}$	75	< 1	97
7	reduced pressinoic acid	CYFQNC-OH	90	*trans*-$[PtCl_2(phen)(en)]^{2+}$	92	< 1	99
				trans-$[PtCl_2(bpy)(en)]^{2+}$	94	< 1	99
8	reduced crustacean cardioactive peptide	PFCNAFTGC-NH_2	90	*trans*-$[PtCl_2(phen)(en)]^{2+}$	95	< 1	99
				trans-$[PtCl_2(bpy)(en)]^{2+}$	94	< 1	99
9	reduced phenypressin	CFFQNCPRG-NH_2	85	*trans*-$[PtCl_2(phen)(en)]^{2+}$	88	< 1	98
				trans-$[PtCl_2(bpy)(en)]^{2+}$	88	< 1	98
10	reduced vasotocin	CYIQNCPKG-OH	84	*trans*-$[PtCl_2(phen)(en)]^{2+}$	90	< 1	96
				trans-$[PtCl_2(bpy)(en)]^{2+}$	90	< 1	97
11	Reduced atriopeptin II (rat, rabbit, mouse)	SSCFGGRIDRIGAQSG LGCNSFR-OH	90	*trans*-$[PtCl_2(phen)(en)]^{2+}$	85	3	98
				trans-$[PtCl_2(bpy)(en)]^{2+}$	82	3	98

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[a, b] Purities of peptides were calculated by the areas of the chromatographic products peaks recorded at 215 nm; Peak areas of Pt(IV)/Pt(II) are not included when calculating the purities of oxidized peptides.

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Figure Captions

Figure 1. HPLC chromatograms of a) peptide 1; b) the reaction between *trans*-$[PtCl_2(phen)(en)]^{2+}$ (2.1 mM) and peptide 1 (1.0 mM) in 10.0 mM of HCl solution; c) the reaction between *trans*-$[PtCl_2(bpy)(en)]^{2+}$ (2.1 mM) and peptide 1 (1.0 mM) in 10.0 mM of HCl solution; d) the reaction between *trans*-$[PtCl_2(CN)_4]^{2-}$ (2.2 mM) and peptide 1 (1.0 mM) in 10.0 mM of HCl solution.

Figure 2. HPLC chromatograms of a) reduced α-conotoxin SI; b) the reaction between *trans*-$[PtCl_2(phen)(en)]^{2+}$ and reduced α-conotoxin SI in 10.0 mM of HCl solution.

Figure 3. HPLC chromatograms of a) mixture of peptide 1 (1.0 mM) and reduced phenypressin (1.0 mM) in 10.0 mM of HCl solution; b) the reaction between *trans*-$[PtCl_2(phen)(en)]^{2+}$ (4.0 mM) and the mixture of peptide 1 (1.0 mM) and eeduced phenypressin (1.0 mM) in 10.0 mM of HCl solution.

Figure 4. Formation of disulfide bond on resin *via* Pt(IV) complex oxidation.

Scheme 1. The proposed reaction mechanism for the formation of peptide disulfide bond *via* Pt(IV) complex oxidation.

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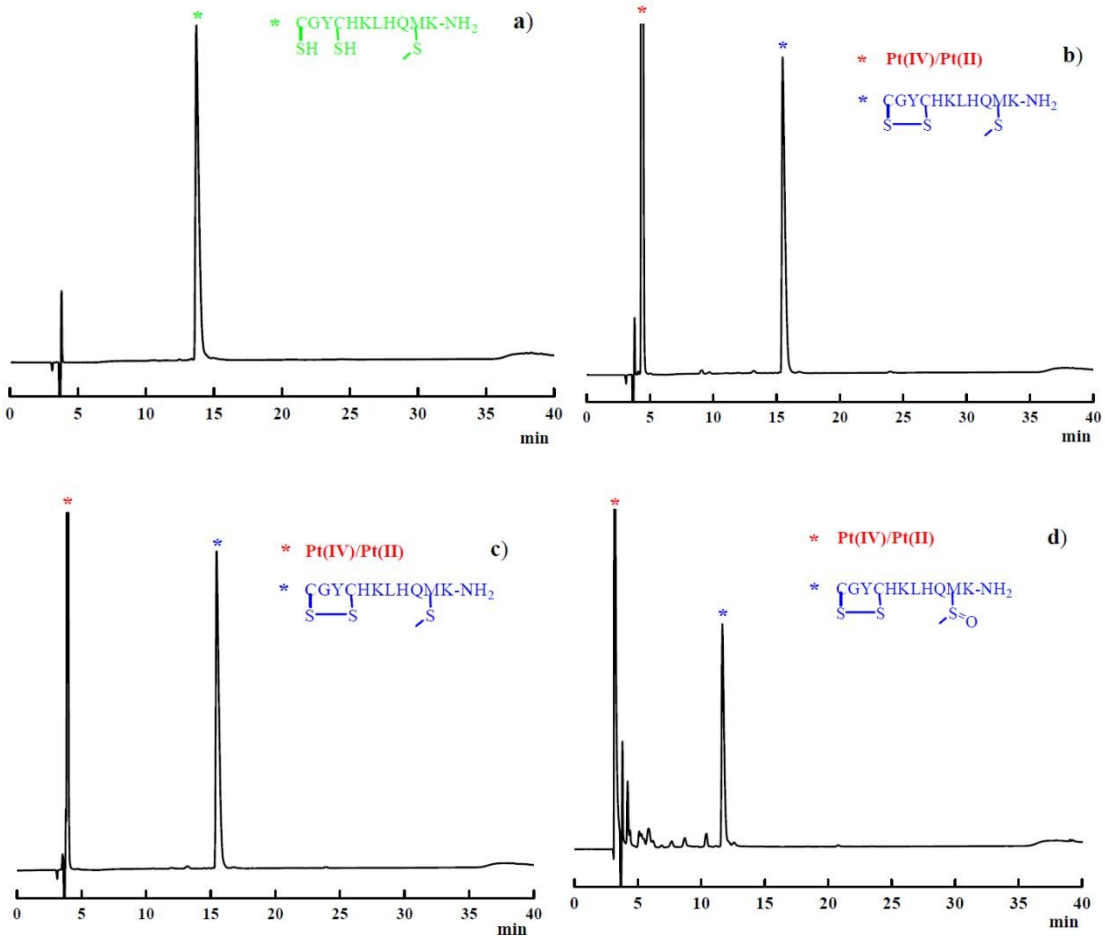

Figure 1

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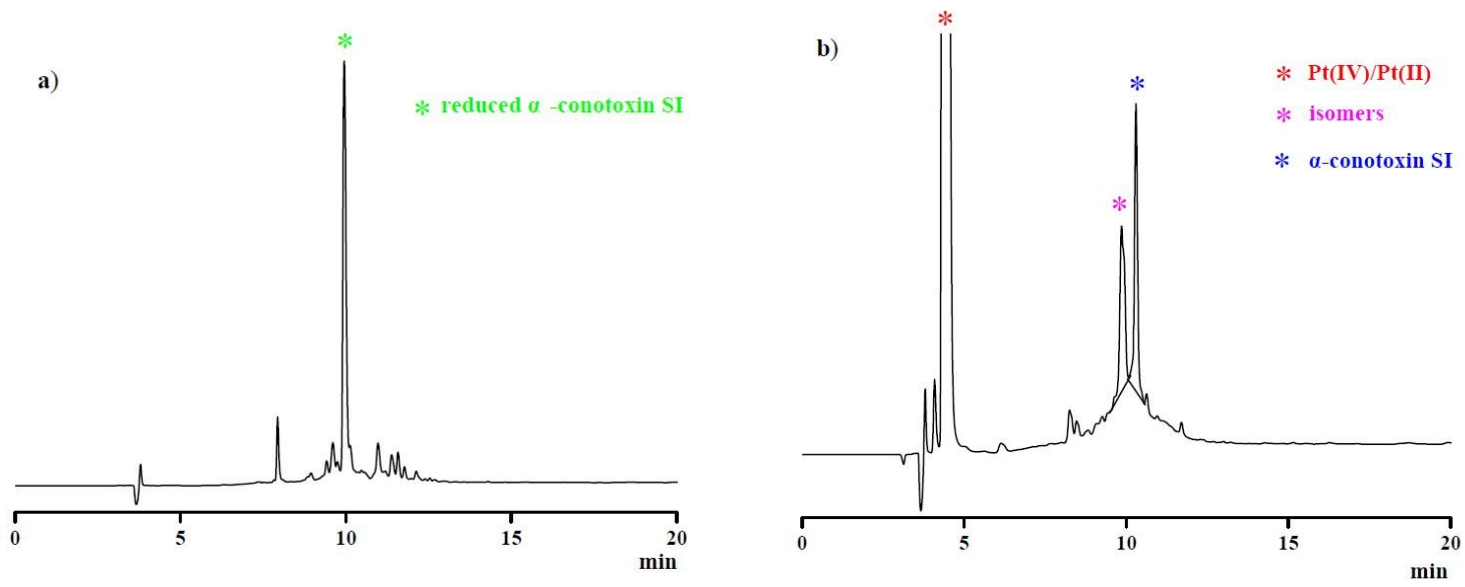

Figure 2

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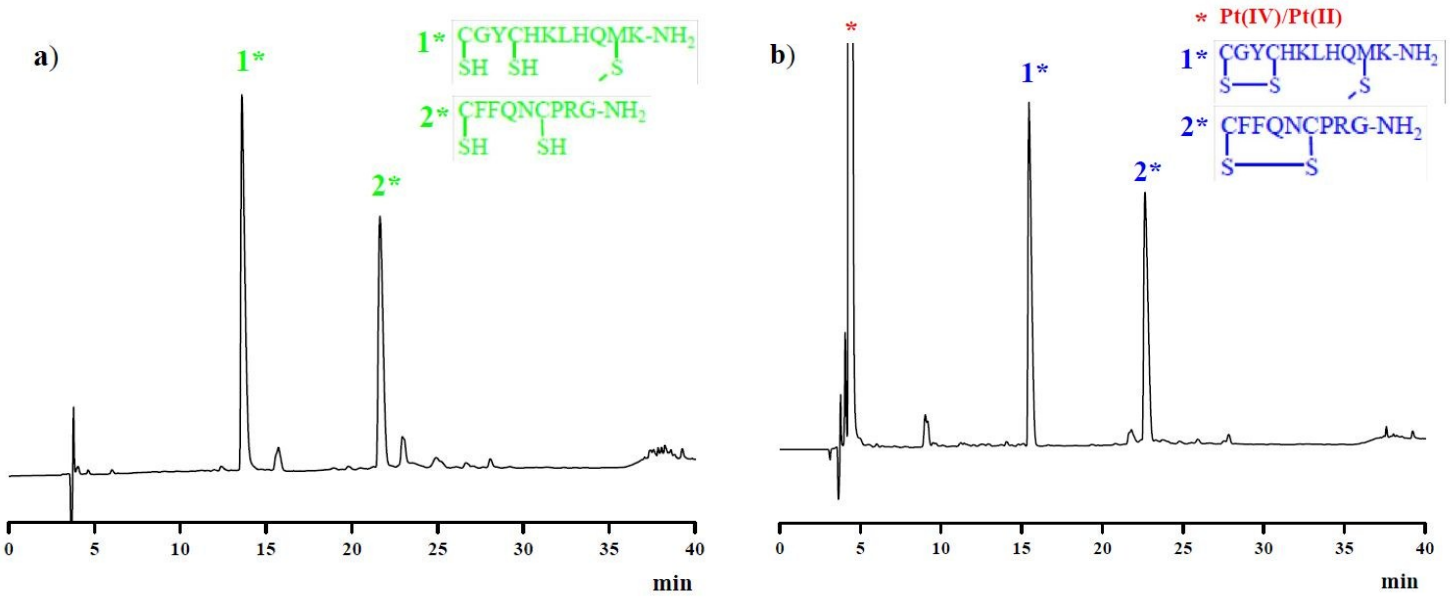

Figure 3

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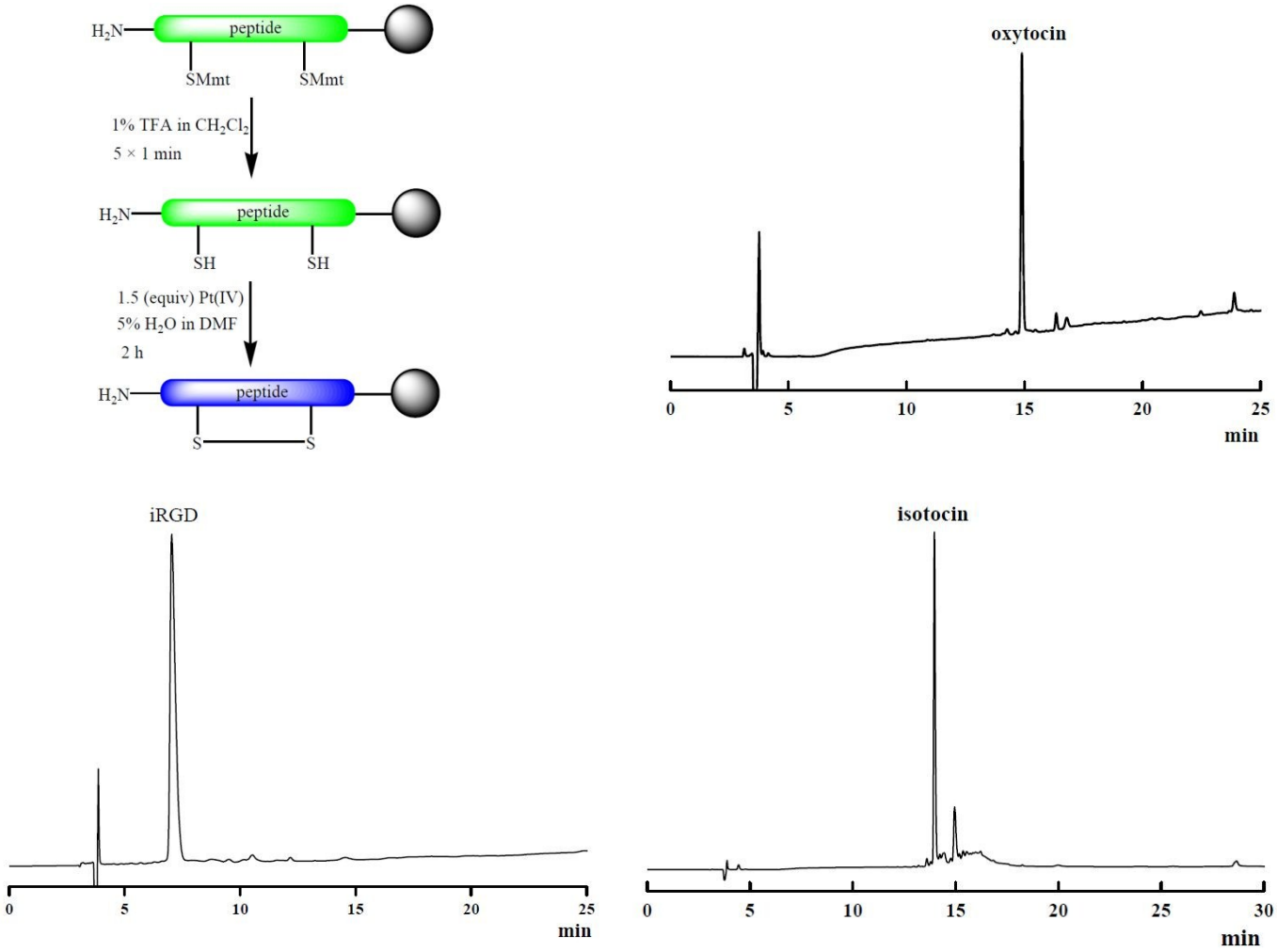

Figure 4

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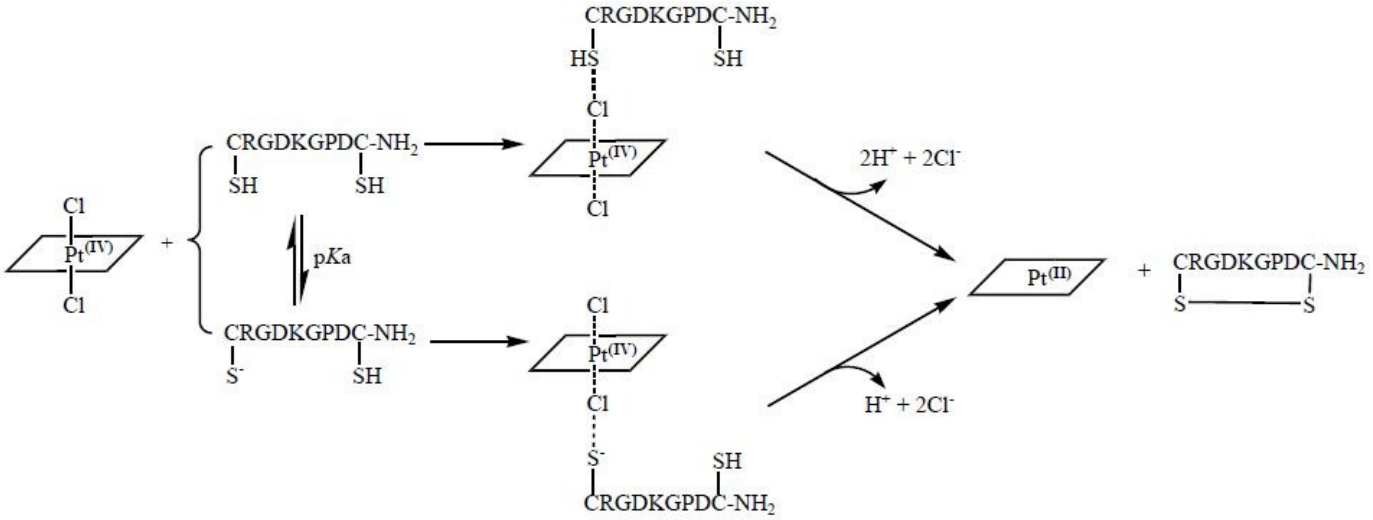

Scheme 1

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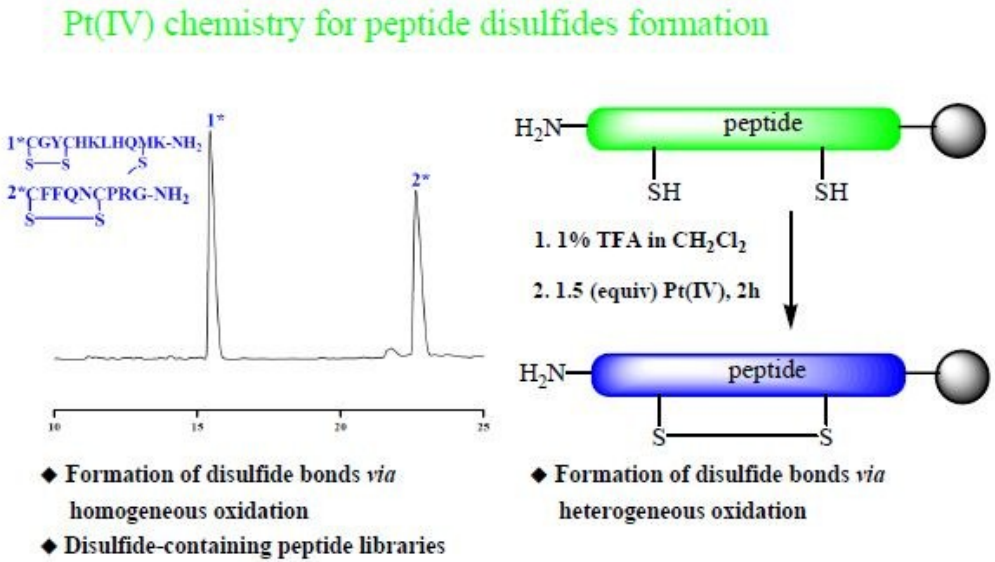
Graphical Abstract



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