Journal of Molecular Catalysis A: Chemical 407 (2015) 194–203

Contents lists available at ScienceDirect

Journal of Molecular Catalysis A: Chemical

journal homepage: www.elsevier.com/locate/molcata

Diazo ester insertion in N—H bonds of amino acid derivatives and insulin catalyzed by water-soluble iron and ruthenium porphyrin complexes (FeTSPPCl) as application of carbenoid transfer in aqueous media

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ARTICLE INFO

Article history:
Received 7 April 2015
Received in revised form 4 June 2015
Accepted 23 June 2015
Available online 8 July 2015

Keywords:
Catalytic NH insertion
Iron porphyrin
Diazo ester
Amino ester
Insulin

ABSTRACT

The metal complex FeTSPPCl (5,10,15,20-tetrakis)-(4-sulfonato-phenyl)-porphyrin-iron(III) chloride is an active catalyst for carbenoid insertion in N—H bonds of aminoacid derivatives in aqueous media. A variety of diazoacetates and methyl diazophosphonate were used as carbenoid precursors. The commercially available iron porphyrin complex can also selectively catalyze alkylation of the *N*-terminus of insulin (chain B).

1. Introduction

The most common bioconjugations are coupling of a small molecule (such as biotin or a fluorescent dye) to a protein. Usual types of bioconjugation chemistry are amine coupling of lysine amino acid residues (typically through amine-reactive succinimidyl esters) and sulfhydryl coupling of cysteine residues (via a sulfhydryl-reactive maleimide) [1]. Generally, the preparation of bifunctional agents involves multistep syntheses, and their subsequent incorporation into biomolecules is often hampered by cross-reactivity or nonspecific interactions with other functional groups present. Thus, the quest for novel, efficient strategies for the synthesis of functional agents, and their incorporation into biomolecules, has led to a burgeoning interest in "metallocarbenoid" chemistry [2].

The catalytic N—H insertion of α-diazocarbonyl compounds is a powerful organic reaction which has various useful applications [3–5]. One of the most successful intramolecular N—H insertions has been the conversion of a penicillin analogue into the carbapenem nucleus via rhodium-catalyzed insertion of a keto carbenoid into the N—H bond of the β-lactam [6]. More generally, this insertion reaction can lead to the synthesis of α-amino esters, dipeptides, and nitrogen-containing heterocycles. The catalytic preparation of optically active amines, recently reported by Zhou et al. [7] is also a nice example of application in asymmetric synthesis. Protein modification through alkylation of the *N*-terminal α-amino acid is also a potentially useful approach such as recently reported by the group of Che [8]. Among the previously reported metal-catalyzed carbenoid transfer, selective tryptophan modification with rhodium carbenoids [9,10] and terminal amino acid modification of proteins with ruthenium porphyrins have been previously explored [11], although in the latter case, a stoichiometric amount of the metalloporphyrin was necessary.

We reported the first success in the use of ruthenium porphyrin catalysts for intermolecular insertion of diazo derivatives into N—H bonds in 1997 [12]. Since then, we [13,14] and others [11,15–18] have found that ruthenium and iron porphyrins are active catalysts, for carbenoid transfer, not only in organic solvents but also in protic solvents and water. Although, there are examples showing reactions with diazoacetonitrile [19] diazomethyl phosphonate [20] and trifluoro diazomethane [21] in organic solvents, ethyl dia-

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http://dx.doi.org/10.1016/j.molcata.2015.06.028
1381-1169/

zoacetate has been mainly used as the carbenoid precursors in water.

In this paper, we report NH insertion of amino esters using various diazo esters [5] as reagents and the site selective modification of terminal NH_2 group of insulin using water-soluble 5,10,15,20-tetrakis-(4-sulfonato-phenyl)-porphyrin-iron(III) chloride (FeTSPPCl) **1** as catalyst (Scheme 1). Iron porphyrin-catalyzed NH insertions have been previously studied in organic solvents [15–18]. Aviv and Gross [18] also reported intermolecular N—H insertion of anilines in water/THF catalyzed by myoglobin. Although, there are many examples showing that 5,10,15,20-tetrakis-(4-sulfonato-phenyl)-porphyrin-iron(III) chloride is a versatile water-soluble catalyst for oxidations in organic syntheses [22] and few other reactions [23,24], its use for carbenoid transfer in water seems to be neglected [25]. Comparative evaluation of reactivity for N-H insertion of different diazo derivatives with iron and ruthenium porphyrins will also be described.

2. Experimental

2.1. General

All reactions were performed under argon and were magnetically stirred. Solvents were distilled from appropriate drying agent prior to use: MeOH from turning Mg. Commercially available reagents (Acros) were used without further purification unless otherwise stated. All reactions were monitored by TLC with Merck pre-coated aluminium foil sheets (Silica gel 60 with fluorescent indicator UV254). Compounds were visualized with UV light at 254 nm. Column chromatographies were carried out using silica gel from Merck (0.063–0.200 mm). UV–vis spectra were recorded on a UVIKON XL from Biotech. MALDI-TOF mass spectra were recorded on microflex Bruker. The analysis were performed on a ThermoFisher LC–MS system (a DionexUltiMate 3000 quaternary RSLC Nano-UPHLC system coupled with a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer). Ethyl diazoacetate and 5,10,15,20-tetrakis-(4-sulfonato-phenyl)-porphyrin-iron(III) chloride are commercially available. 5,10,15,20-tetrakis-(4-sulfonatophenyl)-porphyrin-ruthenium(II) carbonyl was prepared as previously reported [14]. Diisopropyl diazomethylphosphonate was prepared as previously reported [26,27].

2.2. Syntheses of diazo compounds

The synthesis of substituted benzyl diazoacetates was adapted from the method previously reported by Fukuyama [28]. For the synthesis of the 4-(trifluoromethyl)benzyl 2-diazoacetate, 4-(trifluoromethyl)benzyl bromoacetate was first prepared: 4-(trifluoromethyl)benzyl alcohol (548 μL, 4.0 mmol) and $NaHCO_3$ (1.0 g, 12.0 mmol) were dissolved in acetonitrile (20 ml) and bromoacetylbromide (524 μl, 6.0 mmol) was added slowly at 0 °C in a 100 ml round bottom flask. After stirring for 15 min at room temperature, the reaction was quenched with H_2O. The solution was extracted with CH_2Cl_2 three times. The organic phase was washed with brine and dried over $MgSO_4$. The solvent was evaporated, and the residue was used in the next reaction without purification. The 4-(trifluoromethyl)benzyl bromoacetate thus obtained and *N*,*N*′-ditosylhydrazine (272 mg, 8.0 mmol) were dissolved in THF (20 ml) and cooled to 0 °C. DBU (3 ml, 20 mmol) was added dropwise and stirred at room temperature for 10 min. After quenching the reaction by the addition of saturated $NaHCO_3$ solution, this was extracted three times with diethylether. The organic phase was washed with brine, dried over $MgSO_4$ and evaporated to give the crude diazo acetate. Purification of the diazo acetate was performed with neutral silica gel to give 4-(trifluoromethyl)benzyl 2-diazoacetate as a pale yellow oil (897 mg, 90%, two steps). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): δ 4.83 (s, 1H, CH=N_2), 5.25 (s, 2H, CH_2), 7.47 (d, *J* = 8.02 Hz, 2H), 7.63 (d, *J* = 7.63 Hz, 2H). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): δ 46.48 (CH=N_2), 65.56 (CH_2), δ 123.05 (CF_3), 125.65 (CHPh), 128.23 (CHPh), 130.57 (Cq-CF_3), 140.06 (Cq-CH_2), 166.57 (C=O). ^{19}F NMR ($CDCl_3$, 400 MHz, ppm): δ −62.67 (s). HR-MS (*m*/*z*): calculated mass for $C_{10}H_7N_2O_2F_3Na$ [M + Na]$^+$: 267.03573, found *m*/*z*: 267.0356 (0 ppm).

For the synthesis of the 4-(methylsulfide)benzyl 2-diazoacetate, the previous experimental procedure was employed. The expected product was obtained as a yellow oil (yield 88%). ^{1}H NMR ($CDCl_3$, 400 MHz, ppm): δ 2.48 (s, 3H, SCH_3), 4.79 (s, 1H, CH=N_2), 5.16 (s, 2H, CH2), 7.24–7.30 (m, 4H, Ph). ^{13}C NMR ($CDCl_3$, 125 MHz, ppm): δ 15.85 (SCH_3), 46.48 (CH=N_2), 66.28 (CH_2), 126.68 (CHPh), 129.07 (CHPh), 132.72 (Cq), 139.08 (Cq), 166.8 (CO_2). HR-MS (*m*/*z*): calculated mass for $C_{10}H_{10}N_2O_2NaS$ [M + Na]$^+$: 245.03607, found *m*/*z*: 245.0361 (0 ppm).

2.3. General procedure for NH insertion in water

In a typical experiment, the α-amino ester (0.18 mmol) and FeTSPPCl catalyst (1.8 μmol) were placed in a schlenck tube under argon and dissolved in 2 ml of degazed bicarbonate buffer solution (pH 10). Ethyl diazoacetate (0.18 mmol) was then added at room temperature. After 15 min of stirring the reaction was stopped and the mixture was then extracted three times with CH_2Cl_2, dried by $MgSO_4$ and purified by column chromatography on silica gel (CH_2Cl_2/Methanol:97/3). A similar procedure was effective for N—H insertion catalyzed by RuTSPPCO, excepted that the reaction was stopped after 4 h.

2.4. General procedure for NH insertion in organic solvent

In a typical experiment the α-amino ester (0.18 mmol) and FeTPPCl (1.3 mg, 1.8 μmol) were placed in a schlenck tube under argon and dissolved in 2 ml of distilled CH_2Cl_2. NEt_3 (0.27 mmol) was added to the solution. (Ethyl diazoacetate) (0.18 mmol) was then added at room temperature. After 2 min of stirring, the insertion product was purified by column chromatography on silica gel (CH_2Cl_2/Methanol: 95/5).

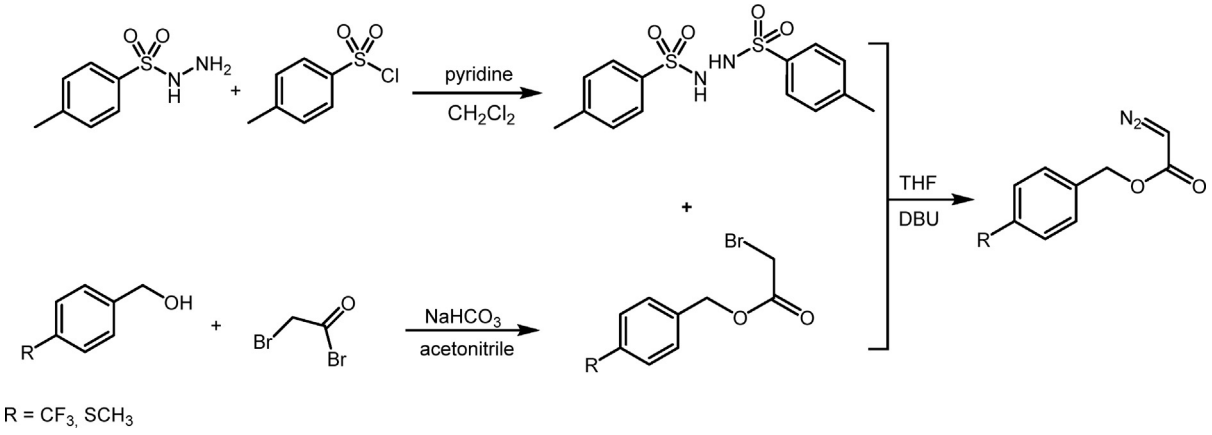
2.5. NH insertion of insulin

In a typical experiment, insulin (5 mg, 0.87 μmol) and 0.2 equiv of TPPSFeCl (0.2 mg, 0.17 μmol) were dissolved in 10 ml of PBS and 5 ml of acetonitrile under nitrogen. 0.32 mg of cobaltocene was first added to the solution and then, the diazo derivative (1.3 mmol) diluted in 10 μl of acetonitrile. The solution was stirred for 1 h. The yield was estimated by MALDI-TOF. After centrifugation, insulin was purified by preparative HPLC and then dried under vacuum to give a white powder.

2.6. Characterization of N—H inserted compounds

2.6.1. Reaction of ethyl diazoacetate with amino esters

Mono-inserted tyrosine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.23 (t, *J* = 7.2 Hz, 3H, CH_3), 2.88–2.98 (m, 2H, CH_2), 3.37 (sys AB, *J* = 17 Hz, 2H, CH_2), 3.57 (t, 6.8 Hz, 1H, CH), 3.67 (s, 3H, OCH_3), 4.14 (q, 7 Hz,2H, CH_2), 6.71 (d, *J* = 8.5 Hz, 2H, CH), 7.02 (d, 8.5 Hz, 2H, CH). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.13 (CH_3 (CO_2Et)), 38.56 (CH_2Ph), 49.11 (CH_2NH), 51.92 (CH_3 (CO_2Me)), 61.02 (CH_2 (CO_2Et)), 62.23 (*CH), 115.51 (CH, Ph), 128.33 (Cq, CH_2Ph), 130.32 (CH, Ph), 154.9 (Cq (PhOH)), 171.75 (Cq (CO_2Et)), 174.22 (Cq (CO_2Me)). HR-MS (*m*/*z*): calculated mass for $C_{14}H_{19}NO_5$ [M + Na]$^+$: 304.29, found *m*/*z*: 304.11(3 ppm)



Scheme 1. Synthesis of benzyl diazoacetate derivatives.

Bis-inserted tyrosine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.25 (t, J = 7.2 Hz, 6H, 2xCH_3), δ 2.91–2.99 (m, 2H, CH_2), δ 3.57 (s, 3H, OCH_3), 3.63 (t, 6.8 Hz, 1H, CH), 3.71 (s, 4H, 2xCH_2), δ 4.14 (q, 7 Hz, 2H, CH_2), δ 6.71 (d, J = 8.5 Hz, 2H, CH), δ 7.02 (d, 8.5 Hz, 2H, CH). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.32 (CH_3 (CO_2Et)), δ 36.37 (CH_2Ph), δ 51.50 (CH_3 (CO_2Me)), δ 52.85 (CH_2N), δ 60.82 (CH_2 (CO_2Et)), δ 67.12 (*CH), δ 115.38 (CH, Ph), δ 129.34 (Cq, CH_2Ph), δ 130.48 (CH, Ph), δ 154.56 (Cq (PhOH)), δ 171.60 (Cq (CO_2Et)), δ 172.47 (Cq) (CO_2Me). HR-MS (m/z): calculated mass for $C_{18}H_{25}NO_7Na$ [M + Na]$^+$: 390.15287, found m/z: 390.1528 (0 ppm).

Mono-inserted tryptophan methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.19 (t, J = 7.1 Hz, 6H, CH_3), 3.12–3,27 (m, 2H, CH_2), 3.39 (sys AB, J = 17 Hz, 2H, CH_2), 3.66 (s, 3H, OCH_3), 3.70 (t, 6.1 Hz, 1H, CH), 4.10 (q, 7.1 Hz, 2H, CH_2), 7.08–7.13 (m, 2H, CH), 7.18 (t, J = 7.3 Hz, 1H, CH), 7.34 (d, J = 8 Hz, 1H, CH), 7.6 (d, J = 7.8 Hz, 1H, CH), δ 8.19 (s, 1H, NH (indole)). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.25 (CH_3(CO_2Et)), 29.27 (CH_2Ph), 49.40 (CH_2N), 52.04 (CH_3 (CO_2Me)), 60.97 (CH_2 (CO_2Et)), 61.32 (*CH), 111.03 (Cq), 111.32 (CH), 118.83 (CH), 119.63 (CH), 122.27 (CH), 123.08 (CH), 127.54 (Cq), 136.35 (Cq), 171.78 (Cq (CO_2Et)), 174.52 (Cq (CO_2Me)). HR-MS (m/z): calculated mass for $C_{16}H_{20}N_2O_4Na$ [M + Na]$^+$: 327.13208; found m/z: 327.1322.

Bis-inserted tryptophan methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.25 (t, J = 7.1 Hz, 6H, 2xCH_3), δ 3.13–3.28 (m, 2H, CH_2), δ 3.57 (s, 3H, OCH_3), 3.76 (s, 4H, 2xCH_2), 3.82 (t, 7.4 Hz,1H, CH), δ 4.14 (q, J = 7 Hz, 2H, CH_2), δ 7.11 (t, J = 7.3 Hz, 1H, CH), 7.17 (t, J = 7.2 Hz, 2H, CH), 7.33 (d, J = 8 Hz, 1H, CH), 7.63(d, J = 7.8 Hz, 1H, CH), δ 7.99 (s, 1H, NH (indole)). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.34 (CH_3 (CO_2Et)), δ 26.58 (CH_2Ph), δ 51.45 (CH_3 (CO_2Me)), δ 52.78 (CH_2N), δ 60.73 (CH_2 (CO_2Et)), δ 65.62 (*CH), δ 111.16 (CH), δ 111.45 (Cq), δ 118.79 (CH), δ 119.51 (CH), δ 122.06 (CH), δ 123.15 (CH), δ 127.61 (Cq), δ 136.15 (Cq), δ 171.61 (Cq (CO_2Et)), δ 172.76 (Cq) (CO_2Me). HR-MS (m/z): calculated mass for $C_{20}H_{26}N_2O_6Na$ [M + Na]$^+$: 413.16886; found m/z: 413.1701.

Mono-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.23 (t, J = 7.2 Hz, 3H, CH_3), 2.94–3.05 (m, 2H, CH_2), 3.36 (sys AB, J = 17 Hz, 2H, CH_2NH), 3.60 (t, J = 6.7 Hz,1H, CH), 3.66 (s, 3H, CH_3), δ 4.14 (q, J = 7.1 Hz, 2H, CH_2), δ 7.19–7.30 (m, 5H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.13 (CH_3 (CO_2Et)), 39.67 (CH_2Ph), 49.32 (CH_2NH), 51.85 (CH_3 (CO_2Me)), 60.99 (CH_2 (CO_2Et)), 62.31 (*CH), 127 (CH, Ph), 128.65 (CH, Ph), 129.32 (CH, Ph), 137.04 (Cq ($PhCH_2$)), 171.72 (Cq (CO_2Et)), 174.21 (Cq) (CO_2Me). HR-MS (m/z): calculated mass for $C_{14}H_{19}NO_5Na$ [M + Na]$^+$: 288,12118; found m/z: 288,1207 (2 ppm).

Bis-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.25 (t, J = 7.1 Hz, 6H, 2xCH_3), 2.99–3.08 (m, 2H, CH_2), 3.57 (s, 3H, CH_3), 3.66–3.70 (m, 1H, CH), 3.72 (s, 4H, 2xCH_2), 4.14 (q, J = 7.1 Hz, 4H, 2xCH_2), 7.17–7.27 (m, 5H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.34 (CH_3 (CO_2Et)), 37.28 (CH_2Ph), 52.87 (CH_2NH), 51.46 (CH_3 (CO_2Me)), 60.75 (CH_2 (CO_2Et)), 66.9 (*CH), 126.69 (CH, Ph), 128.50 (CH, Ph), 129.37 (CH, Ph), 137.50 (Cq ($PhCH_2$)), 171.48 (Cq (CO_2Et)), 172.26 (Cq (CO_2Me)). HR-MS (m/z): calculated mass for $C_{18}H_{25}NO_6Na$ [M + Na]$^+$: 374.15796; found m/z: 374.1590 (3 ppm).

Mono-inserted glycine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.25 (t, J = 7.13 Hz, 3H, CH_3) (CO_2Et), 3.44 (s, 2H, CH_2), 3.46 (s, 2H, CH_2 (CH_2NH)), 3.71 (s, 3H, CH_3 (CO_2Me)), 4.17 (q, J = 7.17 Hz, 2H, CH_2 (CO_2Et)). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.27 (CH_3 (CO_2Et)), 50.05 (CH_2), 50.23 (CH_2), 51.93 (CH_3), 60.95 (CH_2 (CO_2Et)), 171.83 (Cq (CO_2Et)), 172.30 (Cq (CO_2Me)). HR-MS (m/z): calculated mass for $C_7H_{13}NO_4Na$ [M + Na]$^+$: 198,07423; found m/z: 1,980,748 (3 ppm).

Bis-inserted glycine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.24 (t, J = 7.2 Hz, 3H, CH_3) (CO_2Et), 3.63 (s, 4H, CH_2($NHCH_2$)), 3.65 (s, 2H, CH_2(CH_2NH)), 3.69 (s, 3H, CH_3(CO_2Me)), 4.15 (q, J = 7.2 Hz, 2H, CH_2(CO_2Et)). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 14.31 (CH_3 (CO_2Et)), 51.81 (CH_3 (CO_2Me)), 55.10 (CH_2 (CH_2CO_2Me)), 55.29 ($NHCH_2$), 60.80 (CH_2 (CO_2Et)), 170.87 (Cq (CO_2Et)), 171.37 (Cq (CO_2Me)). HR-MS (m/z): calculated mass for $C_{11}H_{19}NO_6Na$ [M + Na]$^+$: 284.11101; found m/z: 284.11111 (0 ppm).

Mono-inserted lysine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.23 (t, 3H, CH_3), 1.45 (m, 4H, 2xCH_2), 1.65 (m, 2H, CH_2), 2.69 (t, 2H, CH_2), 3.36 (sys AB, 17.0 Hz, 2H, CH_2NH), 3.71 (s, 3H, CH_3), 4.15 (q, 2H, CH_2), 4.17 (m, 1H, CH).

Mono-inserted S-H cysteine ethyl ester**:** ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 1.32 (2t, 6H, CH_3), 2.97–3.13 (m, 2H, CH_2C*), 3.26 (s, 2H, CH_2S), 3.79 (m, 1H, C*H), 4.24 (2m, 4H, CH_2) containing also 20% of N-H inserted cysteine.

2.6.2. Reaction of 4-(trifluoromethyl)benzyl 2-diazoacetate with amino esters

Mono-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.93–3.05 (m, 2H, CH_2), 3.42 (sys AB, J = 17.40 Hz, 2H, CH_2NH), 3.61 (t, J = 6.90 Hz, 1H, *CH), 3.65 (s, 3H, OCH_3), 5.16 (s, 2H, OCH_2), 7.17–7.29 (m, 4H, Ph), 7.42 (d, J = 8 Hz, 2H, Ph), 7.60 (d, J = 8 Hz, 2H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 39.67 (CH_2), 49.27 (CH_2), δ 51.99 (CH_3O), 62.23 (*CH), 65.71 (CH_2), 124.09 (q, ^{1}JCF = 272.14 Hz, CF_3), 125.68 (q, ^{3}JCF = 3,78 Hz, Ph), 127.04 (Ph), 128.40 (Ph), 128.66 (Ph), 129.31 (Ph), 130.66 (q, ^{2}JCF = 32.57 Hz, Cq), 136.95 (Cq), 139.59 (Cq), 171.45 (Cq, CO_2CH_2), 174.13 (Cq, CO_2Me). NMR ^{19}F (CDCl3, 376 MHz, ppm): δ −62.685. HR-MS (m/z): calculated mass for $C_{20}H_{20}NO_4F_3Na$ [M + Na]$^+$: 41,812,421; found m/z: 4,181,239 (1 ppm).

Mono-inserted tryptophan methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.07 (s, 1H, NH), δ 3.12–3.28 (m, 2H, CH_2), 3.48 (sys AB, J = 17.38 Hz, 2H, CH_2-NH), 3.66 (s, 3H, CH_3), 3.72 (t, J = 7.08 Hz, 1H, CH), 5.12 (sys AB, J = 6.82 Hz, 2H, CH_2Ph), 7.07 (d, J = 1.99 Hz, 1H, Hindole), 7.13 (t, J = 7.75 Hz, 1H, Hindole), 7.2 (t, J = 7.69 Hz, 1H, Hindole), 7.34 (d, J = 8.11 Hz, 1H, Hindole), 7.38 (d, J = 8.01 Hz, 2H, Ph), 7.59 (d, J = 7.97 MHz, 2H, Ph), 7.60 (d, J = 7.04 Hz, 1H, Hindole), δ 8.12 (s, 1H, NHindole). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 29.27 (CH_2), 49.34 (CH_2), 52.05 (OCH_3), 61.26 (*CH), 65.63 (CH_2), 110.86 (Cq), 111.37 (CHNH), 118.75 (Ph), 119.59 (Ph), 122.24 (Ph), 123.15 (Ph), 124.08 (q, $1J_{CF}$ = 272.04 Hz, CF_3), 125.61 (q, $^3J_{CF}$ = 3.74 Hz, CH), 127.47 (Cq), δ 128.33 (Ph), 130.54 (q, $2J_{CF}$ = 32.42, Cq), 136.36 (Cq), 139.57 (Cq), 171.54 (Cq, CO_2CH_2), 174.48 (Cq, CO_2Me). ^{19}F NMR ($CDCl_3$, 376 MHz, ppm): δ −62.632. HR-MS (m/z): calculated mass for $C_{22}H_{21}N_2O_4F_3Na$ [M + Na]$^+$: 457.1351; found m/z: 457.135 (0 ppm).

Mono-inserted tyrosine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.87–2.96 (m, 2H, CH_2), 3.46 (sys AB, J = 17.4 Hz, 2H, CH_2NH), 3.59 (t, J = 6.60 Hz, 1H, CH), 3.67 (s, 3H, CH_3), δ 5.16 (s, 2H, CH_2OCO), 6.69 (d, J = 8.44, Hz, 2H, PhOH), 7.0 (d, J = 8.44 Hz, 2H PhOH),), 7.4 (d, J = 8.03 Hz, 2H $PhCF_3$), 7.6 (d, J = 8.1 Hz, 2H $PhCF_3$). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 38.59 (CH_2), 49.11 (CH_2), 52.1 (CH_3O), 62.23 (*CH), 65.84 (CH_2), 115.68 (Ph), 124.05 (q, ^{1}JCF = 272.08 Hz, CF_3), 125.65 (q, ^{3}JCF = 3.71 Hz, CH), 128.12 (Cq), 128.37 (Ph), 130.40 (Ph), 130.45 (q, ^{2}JCF = 32.51 Hz, Cq), 139.42 (Cq), 155.15 (Cq), 171.56 (Cq, CO_2CH_2), 174.32 (Cq, CO_2Me). ^{19}F NMR ($CDCl_3$, 376 MHz, ppm): δ −62.668. HR-MS (m/z): calculated mass for $C_{20}H_{20}NO_5F_3Na$ [M + Na]$^+$: 434.11913; found m/z: 434.1187 (1 ppm).

Mono-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 4.00 (s, 2H, CH_2, ($NHCH_2$)), δ 5.26 (s, 2H, CH_2), 6.62 (d, J = 7.71 Hz, 2H, *o*-Ph), δ 6.77 (t, J = 7.34 Hz, 1H, *p*-Ph), 7.20 (t, 2H, J = 7.44 Hz, *m*-Ph), 7.42 (d, J = 8.01 Hz, 2H, Ph), 7.62 (d, J = 8.01 Hz, 2H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 46.02 (CH_2), 66.10 (CH_2), 113.19 (CH), 118.70 (CH), 124.07 (q, ^{1}JCF = 272.17 Hz, CF_3), 125.72 (q, ^{2}JCF = 3.72 Hz, CH), 128.41 (Ph), 129.50 (Ph), 130.73 (q, ^{2}JCF = 32.53 Hz, Cq), 139.41 (Cq), 146.99 (Cq), 171.12 (Cq, CO_2CH_2). NMR ^{19}F ($CDCl_3$, 376 MHz, ppm): δ −62.682. HR-MS (m/z): calculated mass for $C_{16}H_{14}NO_2F_3Na$ [M + Na]$^+$: 332.08743; found m/z: 332.0873 (0 ppm).

2.6.3. Reaction of 4-(methylsulfide)benzyl 2-diazoacetate with amino acid derivatives

Mono-inserted aniline: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.38 (s, 3H, (SCH_3)), 3.83 (s, 2H, (CH_2) $NHCH_2$), 5.06 (s, 2H, CH_2Ph), 6.50 (d, J = 7.69 Hz, 2H,*o*-Ph), 6.65 (t, J = 7.34 Hz, 1H, *p*-Ph), 7.08 (t, 2H, J = 7.42 Hz, *o*-Ph), 7.15 (sys AB, J = 8.65 Hz, 4H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 15.71 (SCH_3), 45.98 (CH_2), 66.76 (CH_2), 113.11 (CH), 118.37 (CH), 126.57 (CH), 129.16 (CH), 129.40 (CH), 132.05 (Cq), 139.31 (Cq), 147.03 (Cq), 171.15 (Cq). HR-MS (m/z): calculated mass for $C_{16}H_{17}NO_2NaS$ [M + Na]$^+$: 310.08777; found m/z: 310.0879 (0 ppm).

Mono-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.01 (s, 1H, NH), 2.5 (s, 3H, CH_3S), 2.96–3.08 (m, 2H, CH_2), 3.43 (sys AB, J = 16.34 Hz, 2H, CH_2NH), 3.64 (t, J = 6.81 Hz, 1H, CH), 3.67 (s, 3H, CH_3O), 5.1 (s, 2H, CH_2O), 7.2–7.31 (m, 9H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 15.78 (CH_3S), 39.57 (CH_2), 49.24 (CH_2), 51.89 (CH_3O), 62.17 (*CH), 66.32 (CH_2), 123.90 (Ph), 126.60 (Ph), 126.94 (Ph), 128.58 (Ph), 129.120 (Ph), 129.26 (Ph), 132.26 (Cq), 136.94 (Cq), 139.13 (Cq), 171.52 (Cq, CO_2CH2), 174.08 (Cq, CO_2Me). HR-MS (m/z): calculated mass for $C_{20}H_{23}NO_4NaS$ [M + Na]$^+$: [M + Na]+: 396.12455; found m/z: 396.1248 (1 ppm).

Mono-inserted tryptophan methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.93 (s, 1H, NH), 2.47 (s, 3H, CH_3S), 3.15–3,27 (m, 2H, CH_2), 3.44 (sys AB, J = 16.35 Hz, 2H, CH_2NH), 3.64 (s, 3H, CH_3O), 3.69 (t, J = 6.08 Hz, 1H, CH), 5.03 (sys AB, J = 5.12 Hz, 2H, CH_2Ph), 7.09 (d, J = 2.02 Hz, 1H, Hindole), 7.12 (t, J = 7.78 Hz, 1H, Hindole), 7.18 (t, J = 7.29 Hz, 1H, Hindole), 7.35 (d, J = 8.03 Hz, 1H, Hindole), 7.6 (d, J = 7.84 Hz, 1H, Hindole), 8.07 (s, 1H, NHindole). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 15.73 (CH_3S), 29.21 (CH_2), 49.35 (CH_2), 52.0 (CH_3O), 61.23 (*CH), 66.29 (CH_2), 110.71 (Cq), 111.35 (CH), 118.69 (CH), 119.48 (CH), 122.11 (CH), 123.19 (CH), 126.53 (CH), 127.43 (Cq), 129.1 (CH), 132.23 (Cq), 136.3 (Cq), 139.06 (Cq), 171.66 (Cq, COOCH2), 174.48 (Cq, CO_2Me). HR-MS (m/z): calculated mass for $C_{22}H_{24}N_2O_4NaS$ [M + Na]$^+$: 435.13545; found m/z: 435.1357 (1 ppm).

Mono-inserted tyrosine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 2.51 (s, 3H, CH_3S), 2.9–3.02 (m, 2H, CH_2), 3.45 (AB sys, J = 16.48 Hz, 2H, CH_2NH), 3.61 (t, J = 6.72 Hz, 1H, CH), 3.7 (s, 3H, CH_3O), 5.10 (s, 2H, CH_2), 6.72 (d, J = 8.37 Hz, 2H, PhOH), δ 7.04 (d, J = 8.33 Hz, 2H, PhOH), δ 7.24–7.28 (m, 4H, PhSMe). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 15.73 (CH_3S), 38.59 (CH_2), 49.17 (CH_2), 52.05 (CH_3O), 62.24 (C*H), 66.51 (CH_2), 115.65 (Ph), 126.57 (Ph), 128.13 (Cq), 129.14 (Ph), 130.37 (Ph), 132.1 (Cq), 139.14 (Cq), δ 155.13 (Cq), 171.68 (Cq, CO_2CH_2), 174.3 (Cq, COOMe). HR-MS (m/z): calculated mass for $C_{20}H_{23}NO_5NaS$ [M + Na]$^+$: [412.11946]; found m/z: 412.1195 (0 ppm).

2.6.4. Reaction of diisopropyl diazomethylphosphonate with amino acid derivatives

Mono-inserted aniline: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.32 (d, J = 6.2 Hz, 6H, 2xCH_3), 1.37 (d, J = 6.2 Hz, 6H, 2xCH_3), 3.46 (d, JHP = 13.7 Hz, 2H, CH_2), 3.96 (s broad, 1H, NH), 4.73–4.82 (m, J_{HH} = J_{HP} = 6.3 Hz, 2H, CHOP), 6.68 (d, J = 7.8 Hz, 2H, Ph), 6.77 (t, J = 7.3 Hz, 1H, Ph), 7.21 (t, J = 7.5 Hz, 2H, Ph). ^{13}C NMR, HMBC (125 MHz, $CDCl_3$, ppm): δ 23.94 (d, $^3J_{CP}$ = 4.7 Hz, 2xCH_3), 24.06 (d, $^3J_{CP}$ = 3.8 Hz, 2xCH_3), 41.03 (d, $^1J_{CP}$ = 156.13 Hz, $NHCH_2$), 71.18 (d, $^2J_{CP}$ = 6.72 Hz, 2xCHOP), 113.26 (*o*-Ph), 118.37 (*p*-Ph), 129.19 (*m*-Ph), 147.67 (d, $^3J_{CP}$ = 3.8 Hz, Cq). ^{31}P NMR (162 MHz, $CDCl_3$, ppm): δ 22.68 (s). HRMS (m/z): calculated mass for $C_{13}H_{22}NO_3NaP$ (M + Na)$^+$: 294.1235; found m/z: 294.1236 (0 ppm).

Mono-inserted tryptophan methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.22 (t, J = 6.44 Hz, 6H, 2xCH_3), δ 1.26 (t, J = 6.59 Hz, 6H, 2xCH_3), δ 2.79 (t, J = 14.37 Hz, 1H, CH_2), 3.04 (t, J = 12.94 Hz, 1H, CH_2), 3.08–3.21 (m, 2H, CH_2 (tryp)), 3.63 (s, 3H, CH_3 (OCH_3)), 3.73 (t, J = 6.39 Hz, 1H, *CH), 4.6–4.7 (m, J_{HH} = J_{HP} = 6.3 Hz, 2H, CHOP), 6.99 (s, 1H, Hindole), 7.08 (t, 1H, 7.38 Hz, Ph), 7.15 (t, 1H, J = 7.76 Hz, Ph), 7.33 (d, 1H, J = 8.1 Hz, Ph), 7.57 (d, 1H, 7.88 Hz, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 23.96 (2d, J = 5.09, 5.78 Hz, 2xCH_3), 24.12 (d, J = 3.74 Hz, 2xCH_3), 29.13 (CH_2, (tryp)), 44.47 (d, $^1J_{CP}$ = 158.17 Hz, $NHCH_2$), 51.86 (OCH_3), 62.82 (d, $^3J_{CP}$ = 15.44 Hz, *CH), 70.78 (d, ^2J = 1.8 Hz, CH), 70.94 (s, CH), 110.68 (Cq indole), 111.38 (CH indole), 118.71 (CH indole), 119.35 (CH indole), 121.95 (CH indole), 123.20 (CH indole), 127.54 (Cq indole), 136.39 (Cq indole), 174.55 (Cq (CO)).^{31}P NMR (162 MHz, $CDCl_3$, ppm): δ 23.13 (s). HR-MS (m/z): calculated mass for ($C_{19}H_{29}N_2O_5NaP$) [M + Na]$^+$: 419.1711; found: 419.1712 (0 ppm).

Mono-inserted tyrosine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.22 (t, J = 5.81 Hz, 6H, CH_3), 1.26 (d, J = 6.18 Hz, 6H, CH_3), 2.74 (t, J = 14.38 Hz, 1H, CH_2), 2.77–2.93 (m, 2H, CH_2 (tyrosine)), 3.01 (t, J = 11.85 Hz, 1H, CH_2), 3.59 (t, J = 6.58 Hz, 1H, *CH), 3.65 (s, 3H, OCH_3), 4.58–4.69 (m, J_{HP} = J_{CP} = 6.01 Hz, 2H, CHOP), 6.74 (d, 2H, 8.33 Hz, Ph), 6.96 (d, 1H, 8.33 Hz, CH (Ph). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 23.96 (2d, $^3J_{CP}$ = 4.86 Hz, 2xCH_3), δ 24.1 (d, $^3J_{CP}$ = 3.63 Hz, CH3), 38.67 (CH_2 (tyrosine)), 44.32 (d, $^1J_{CP}$ = 160.04 Hz, $NHCH_2$), 51.86 (OCH3)), 63.71 (d, $^3J_{CP}$ = 14.96 Hz, *CH), 71.42 (2d, $^2J_{CP}$ = 6.84 Hz, CH) 115.58 (Ph), 127.54 (Cq, Ph), 130.24 (Ph), 156.1 (Cq), 174.41 (Cq (CO_2Me)). ^{31}P NMR (162 MHz, $CDCl_3$, ppm): δ 22.93 (s). HR-MS (m/z): calculated mass for ($C_{17}H_{28}NO_6NaP$) [M + Na]$^+$: 396.1552; found: 396.1553 (0 ppm).

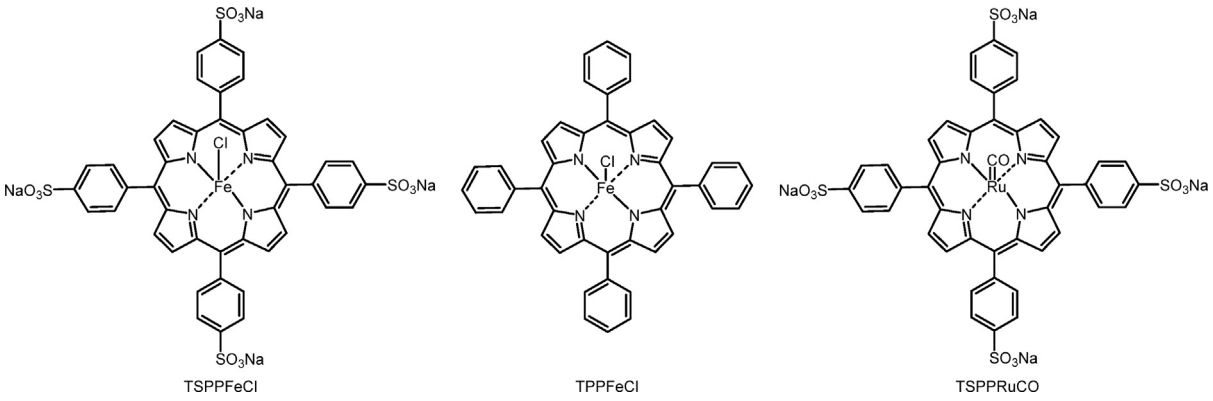


Fig. 1. Structure of the catalysts.

Mono-inserted phenylalanine methyl ester: ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 1.24 (t, J = 7.16 Hz, 6H, 2xCH_3), 1.27–1.29 (m, 6H, 2xCH_3), 1.71 (s large, 1H, NH), 2.76 (t, 1H, CH_2 ($NHCH_2$)), 2.89–3.02 (m, CH_2 (Phenylala.) +1CH_2 ($NHCH_2$)), 3.64 (t, 6.77 Hz, 1H, *CH), 3.67 (s, 3H, (OCH_3)), 4.61–4.70 (m, 2H, CH, 7.17–7.28 (m, 5H, Ph)). ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 24.06 (2d, $3J_{CP}$ = 4.9 Hz, 2xCH3(isopropyl)), 24.2 (2d, $3J_{CP}$ = 2.57 Hz, 2xCH_3), 39.56 (CH_2 (phenylala.)), 45.29 (CH_2, $NHCH_2$), 51.88 (OCH_3)), 63.66 (d, $3J_{CP}$ = 15.42 Hz, *CH), 70.91 (2d), $^2J_{CP}$ = 6.52, 6.93 Hz, 2xCH, 126.88 (Ph), 128.56 (Ph), 129.36 (Ph), 137.23 (Cq), 174.28 (Cq (CO_2Me)). NMR ^{31}P (162 MHz, $CDCl_3$, ppm): 22.73 (s). HR-MS (m/z): calculated mass for $C_{17}H_{28}NO_5PNa$ [M + Na]$^+$: 380.16028; found m/z: 380.1598 (1 ppm).

3. Results and discussion

3.1. Synthesis of diazo derivatives

Besides ethyl diazoacetate and diisopropyl diazomethylphosphonate, two substituted benzyl diazo acetates were also investigated in order to introduce SMe and trifluoromethyl groups. A tosyl group was employed for the synthesis due the availability of inexpensive *p*-toluenesulfonyl hydrazide. The first step was the preparation of 4-(trifluoromethyl)benzylbromoacetate and 4-(methylsulfide)benzylbromoacetate from 4-(trifluoromethyl)benzyl alcohol and 4-(methylsulfide)benzyl alcohol, respectively. Finally, these bromoacetates underwent an easy conversion to the corresponding diazo acetates upon treatment with *N*,*N*′-ditosylhydrazine and DBU at 0 °C, following the protocol previously reported by Fukuyama [28]. The preparation of 4-(trifluoromethyl)benzyl 2-diazoacetate proceeded in a 90% yield, while that of 4-(methylsulfide)benzyl 2-diazoacetate in 88% yield. These diazoacetates were characterized by ^{1}H and the latter by ^{19}F NMR spectroscopy. We note the presence of a characteristic peak at 4.83 ppm and 4.79 ppm (broad singlet) corresponding to CH neighboring N_2 for the CF_3 and SCH_3 derivatives, respectively.

3.2. NH insertion of amino acid esters by ethyl diazoacetate in water

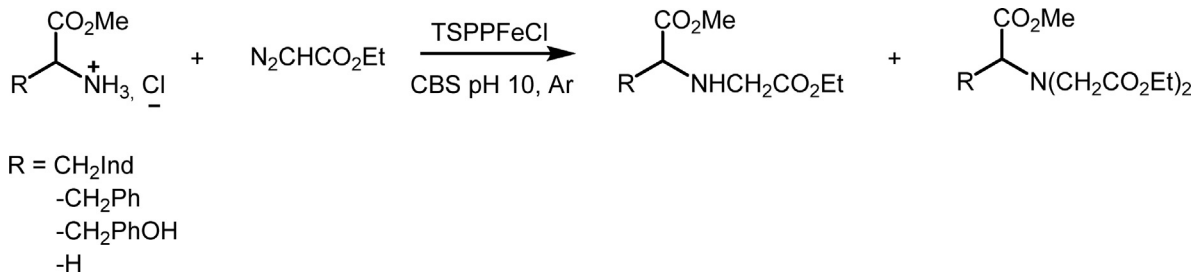
The insertion of electrophilic carbenes into the N—H bonds of α-amino acids and α-amino acid esters could be a powerful method for *N*-alkylating this important class of compounds. However there are several drawbacks and the regioselectivity of the reaction still appears as a difficult problem to solve. This is due to numerous possible reactive sites such as acidic groups, amino groups, thiol groups, and phenol groups. To shine some light on this aspect, we first investigated the regioselectivity of the reaction catalyzed by 5,10,15,20-tetrakis-(4-sulfonato-phenyl)-porphyrin-iron(III) chloride (FeTSPPCl) (Fig. 1) in water.

The results are summarized in Table 1. Thus, FeTSPPCl catalyzed decomposition of 1 equiv of ethyl diazoacetate in the presence of tyrosine methyl ester in dichloromethane resulted in the formation of mono *N*-alkylated amino esters in 71% yield, containing 14% of bis-inserted compound (Table 1, entry 4). A similar result was obtained for tryptophan with 66% yield (Table 1, entry 3). In this case 18% of insertion was the bis-inserted compound (Scheme 2). To extend the scope of this N—H insertion, the reaction of a number of amino acid derivatives with ethyl diazoacetate in the presence of TSPPFeCl as catalyst was studied at room temperature (25 °C). In all these experiments, N-H insertion is the major reaction yielding alkylated products with moderate yield (43–71%), usually obtained with a small amount of bis-inserted compounds. Glycine is an exception since, in this case, the bis-inserted compounds was obtained with 32% yield (Table 1, entry 6). A complete regioselectivity is obtained with tyrosine, since no O—H insertion was detected. With tryptophan methyl ester, only α-amino group was reactive since no insertion was detected in the NH group of indole ring. Because the difference of pK_a values of the *N*-terminal group (∼7.6–8) and the NH_2 group in the side chain of lysine residue (10.0–10.2), this reaction (81% yield) can also be optimized to maximize the selectivity for the *N*-terminal insertion by the reaction being performed under weakly basic conditions (pH 9). Actually cysteine ethyl ester was also tested to detect any selectively. With this amino ester, both N—H insertion and S—H insertion

Table 1
Ethyl diazoacetate insertion in N—H bonds of amino esters catalyzed by FeTSPPCl and RuTPPSCO in water.[a]

	Catalyst	Substrate	Time	Yield	Mono/bis ratio (%)[b]
1	–	R = tryptophan	24	0	–
2	TSPPFeCl	Aniline	15 min	43	100:0
3	TSPPFeCl	R = tryptophan	15 min	66	82:18
4[c]	TSPPFeCl	R = $-CH_2$PhOH	15 min	71	86:14
5	TSPPFeCl	R = $-CH_2$Ph	15 min	62	83:17
6	TSPPFeCl	R = H	15 min	43	68:32
7	TPPFeCl	R = tryptophan	2 min	82	90:10
8	TPPFeCl	R = $-CH_2$Ph	2 min	80	89:11
9	TSPPFeCl	Mono-inserted tryp.	24 h	Traces	
10[d]	TSPPFeCl	Mono-inserted tryp.	1 h	48	
11[e]	TSPPRuCO	R = tryptophan	4 h	56	80:20

Reaction conditions:
[a] Catalyst/substrate/diazo mixture (1:100:100) in 1 ml of CBS (pH 10) at 25 °C.
[b] determined by ^{1}H NMR.
[c] Solvent: EtOH/CBS (50/50) (pH 10).
[d] 20 equiv of sodium dithionite vs catalyst are added.
[e] Amino ester and diazo dissolved in ethanol are progressively added to a buffer solution (CBS pH 10) containing the catalyst.



Scheme 2. Catalytic insertion of ethyl diazoacetate in N—H bond of amino esters.

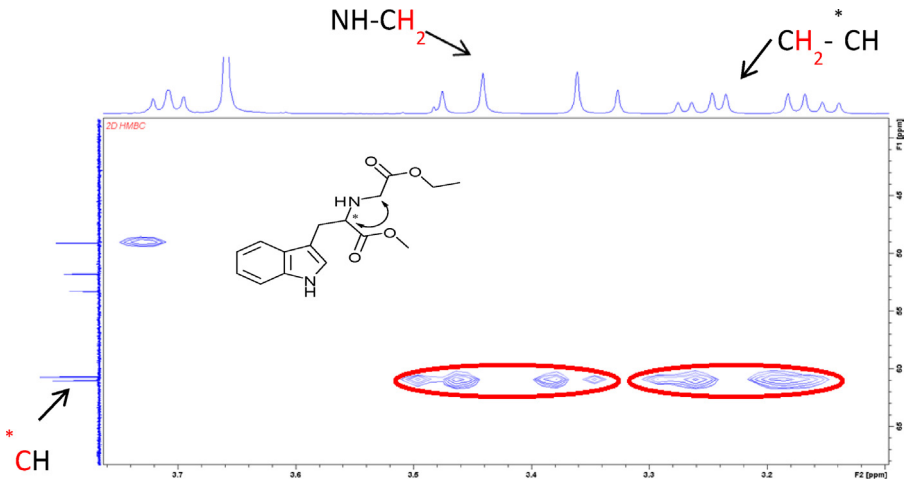


Fig. 2. ^{1}H-^{13}C HMBC spectrum of ethyl diazoacetate insertion in N—H terminus bond of tryptophan.

were observed, the latter being largely predominant (>70%, from ^{1}H NMR) using one equivalent of the diazo derivative. Unfortunately, we were not able to separate the two compounds.

The diazo acetates were added to the amino acids in a water–methanol solution containing the catalyst under argon. Argon was used as a protecting atmosphere due to a possible formation of reduced iron(II) in the catalytic cycle [17]. The reactions were followed by TLC and were left until the complete disappearance of the diazo acetates. We note that traces of the amino acids remained when the reactions were over and this is due to the formation of carbene dimers. The products were characterized by NMR spectroscopy. We note in the ^{1}H NMR spectrum, the presence of characteristic AB systems (~3.4 ppm), which are due to the two protons on the carbon next to the amine function. For example, the spectrum resulting after diazo insertion in tryptophan is shown Fig. 2. This result was observed for all chiral amino esters and is related to the presence of the asymmetric carbon atom. With glycine, only one singlet was noted at 3.6 ppm.

Although, there are a large range of strategies and reagents for coupling amino acids available, racemization of amino acids is still a major problem in these reactions [29]. Due to the basic pH of the carbonate buffer solution, it was necessary to verify the absence of racemization during the insertion process [29,30]. Phenylalanine methyl ester was used as substrate to check a possible racemization. After ethyl diazoacetate coupling, the enantiomeric and the racemic forms of phenylalanine were analyzed separately on HPLC column with chiral phase as the stationary phase. The results are summarized in Fig. 3. The chiral analysis gave 50% of each isomer after insertion with racemic phenylalanine whereas a pure enantiomer was obtained with L-phenylalanine, as expected for a reaction without any racemization.

3.3. NH insertion of amino acid esters by benzyl diazocetate derivatives

We first investigated the reaction of 4-(thiomethyl)benzyl diazoacetate and 4-(trifluoromethyl)benzyl diazoacetate with aniline

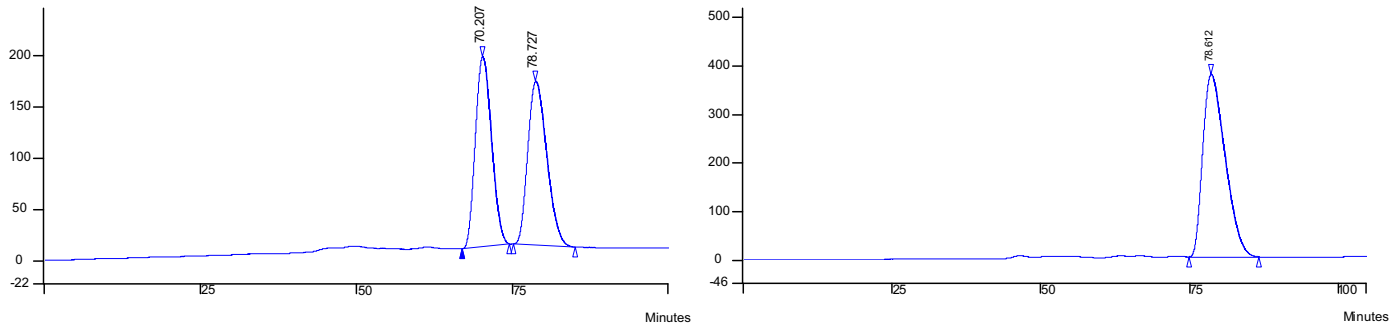
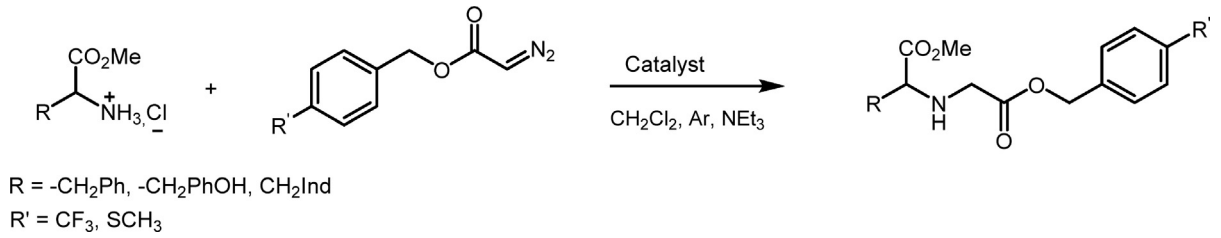
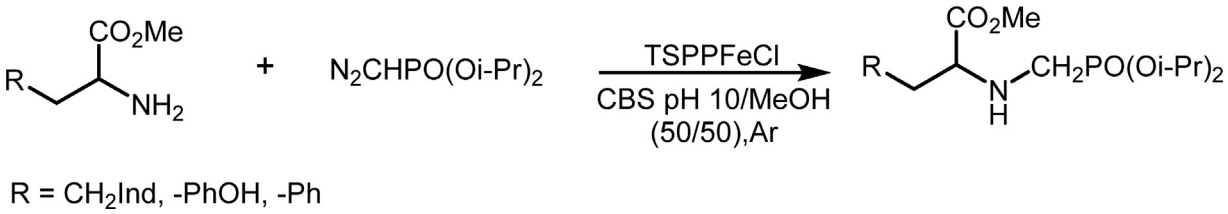


Fig. 3. HPLC chromatograms of racemic (A) and optically active (B) phenylalanine N—H insertion with ethyl diazoacetate.



Scheme 3. Catalytic insertion of benzyl diazoacetate derivatives in N—H bond of amino esters.



Scheme 4. Catalytic insertion of diisopropyl diazomethylphosphonate derivatives in N—H bond of amino esters.

and amino esters (Table 2) in organic solvent. In particular, we wanted to check if these reactions can tolerate the presence of sulfur atom in the diazo compound. The insertion reactions are shown in Scheme 3 and the results reported in Table 2. In these case, the catalyst used for these reaction was TPPFeCl (Fig. 1). The reactions are very fast (<2 mn). The yields obtained are quite high (87–88%) with the diazoester bearing the CF_3 groups if we except the results obtained with tyrosine (66%) (Table 2, entry 4) due probably to solubility difficulty in CH_2Cl_2. Aniline gave also a good yield (97%) (Table 2, entry 1). In contrast, the yields are only moderate with the diazo bearing the SMe group (50–67%) and some amount of the starting material was recovered (Table 2, entries 5–8). A possible interaction of the sulfur atom with iron may inhibit the catalyst, and consequently may explain the low conversion. As previously indicated with ethyl diazoacetate, all the insertion products were characterized by NMR, showing also an AB system for CH_2 group in alpha position of the nitrogen.

To examine whether these diazo derivatives could be used for intermolecular N—H insertion of amino acid derivatives in protein, the catalytic reaction was also realized with water soluble tetra-*p*-(SO_3-)phenyl metalloporphyrins (Fe and Ru). These two metalloporphyrins were chosen because they are easily prepared and commercially available. With the goal of developing protein–compatible reaction conditions, we selected methanol as a co-solvent for diazo ester and amino acid derivative solubilities. All the reactions with benzyl diazoacetate derivatives are summarized in Table 3. First, it should be noted that the yields are only moderare (40–60%) using a small excess of the diazo derivatives (2 equiv) vs substrate. In these water conditions, insertion of the α-amino group was only observed with lysine methyl ester. As both ruthenium and iron porphyrins react with nitrogen compounds to form stable adducts, their decreased activity (by comparison with cyclopropanation reactions) [31] can be attributed to competitive coordination between the amino substrate and ethyl diazoacetate onto the metal. Since, axial Ru—N bond seems much stronger than Fe—N bond, iron catalysts are more efficient but less tolerant to functional group such as thiol groups.

In order to give a clearer characterization of the products, a HMBC spectrum was done for the NH insertion reaction of tyrosine with 4-(methylsulfide)benzyl 2-diazoacetate. This spectrum shows the coupling between a carbon and hydrogen present on the neighboring carbon. We note a coupling between the protons of CH_2 (CH_2NH) and the asymmetric carbon of the amino acid. Also, these protons are coupled to the carbon of the ester function. This result proves the formation of the NH—CH_2 bond.

3.4. NH insertion of amino acid esters by diisopropyl diazomethylphosphonate

Intramolecular N—H insertion of diazomethylphosphonate derivatives was first developed for the synthesis of antibiotic carbapenems [32]. Since then Aller et al. [33] have developed significant diazomethylphosphonate-insertion reactions using rhodium complexes as catalysts for a carbenoid approach to peptide synthesis [34]. Many other biological applications have been related to the use of *N*-(phophomethyl) aminoacids such as shikimate pathway inhibitors [35] and selective thrombin inhibitors [36].

Table 2
Insertion of 4-(trifluoromethyl)benzyl diazoacetate (F) and 4-(thiomethyl)benzyl diazoacetate (S) in N—H bond of amino esters catalyzed by TPPFeCl in CH_2Cl_2.

	Diazo	Catalyst	Substrate	Time	Yield (%)[a]
1	F	TPPFeCl	Aniline	2 min	97
2	F	TPPFeCl	Tryptophan	2 min	88
3	F	TPPFeCl	Phenylalanine	2 min	87
4	F	TPPFeCl	Tyrosine	2 min	66
5	S	TPPFeCl	Aniline	2 min	62
6	S	TPPFeCl	Tyrosine	2 min	50
7	S	TPPFeCl	Tryptophan	2 min	67
8	S	TPPFeCl	Phenylalanine	2 min	63

[a]Reaction conditions: a solution of substrate/diazo/(TPPFeCl) mixture (1:1:0.01) in 1 ml of CH_2Cl_2 placed under argon at 25 °C was stirred for 2 min.

Table 3
Insertion of 4-(trifluoromethyl)benzyl diazoacetate (F) and 4-(thiomethyl)benzyl diazoacetate (S) in N—H bond of aminoesters catalyzed by TPPSFeCl in water/methanol.

	Diazo	Catalyst	Amino ester	Time	Yield (%)
1	F	TPPSFeCl	Tryptophan	10 min	79
2	F	TPPSFeCl	Phenylalanine	10 min	81
3	F	TPPSFeCl	Tyrosine	10 min	51
4[a]	F	TPPSRuCO	Tryptophan	4 heures	45
5	S	TPPSFeCl	Tyrosine	10 min	33
6	S	TPPSFeCl	Tryptophan	10 min	51

Reaction conditions: a solution of substrate/diazo/catalyst mixture (1:1:0.01) in 1 ml of MeOH/CBS pH (10) (1/1) placed under argon was stirred at 25 °C.

[a] IDiazo and amino ester dissolved in MeOH were added to the buffer solution ((CBS pH 10) containing the catalyst and stirred for 4 h).

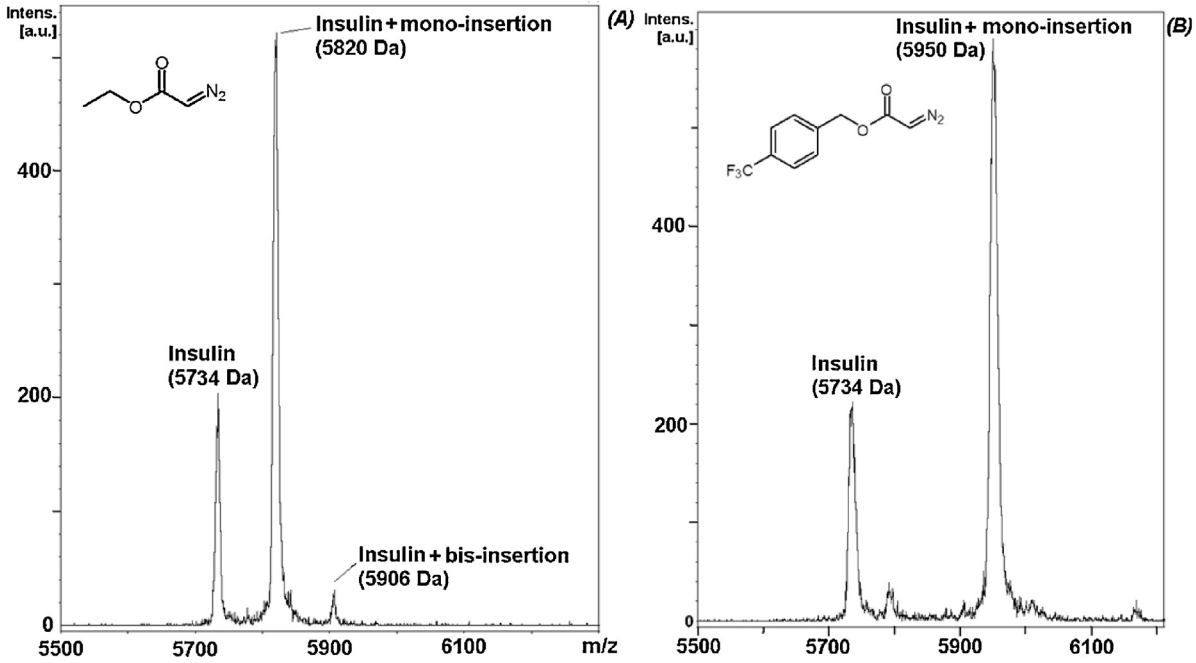


Fig. 4. MALDI-TOF of coupling ethyl diazoacetate (A) and 4-(trifluoromethyl)benzyl diazoacetate (B) to bovine insulin.

Table 4
Insertion of diisopropyl diazomethylphosphonate in N—H bond of amino esters catalyzed by TSPPFeCl in water/methanol.

	Substrate	Yield (%)
1	Tryptophan	90
2	Tyrosine	92
3	Phenylalanine	81
4	Aniline	87
5[a]	Aniline	96

Reaction conditions: a solution of substrate/diazo/catalyst mixture (1:1:0.01) in 2 ml of (MeOH:CBS pH 10) (50:50) was stirred at 25 °C for 1 h.
[a] Solvent: CH_2Cl_2, catalyst: TPPFeCl.

We previously reported the catalytic effectiveness of ruthenium porphyrins for carbenoid generation in reactions of diisopropyl diazomethylphosphonate with alkenes (cyclopropanation) [37] and allylic amine (N—H insertion) [38]. We herein report the N—H insertion of diazophosphonate into amino acid derivatives catalyzed by FeTSPPCl (Scheme 4). The results are summarized in Table 4. In a typical reaction, catalytic insertions into N—H bonds were run in in water/methanol solution at room temperature under argon atmosphere with a substrate:diazo:catalyst ratio of 100:100:1. As shown in Table 4, the coupling products are formed with good yield 81–96% both for aniline and amino acid derivatives without detection of dimerization. Again, no OH insertion was detected for tyrosine.

3.5. NH insertion of insulin by diazo derivatives in water-acetonitrile mixture

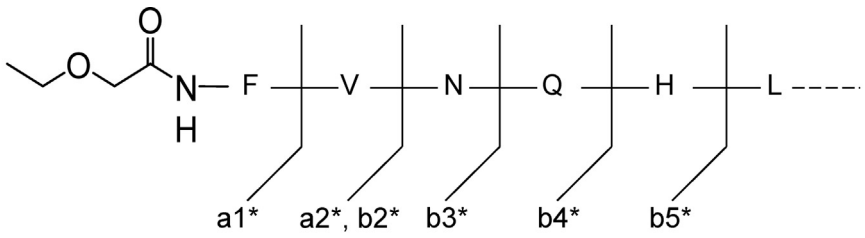
Insulin is a polypeptide hormone that is critical for the metabolism of glucose. The insulin monomer consists of two chains, a 21-residue A chain and a 30-residue B chain, linked by a pair of disulfide bonds, A7—B7 and A20—B—19; an additional intrachain disulfide bond links A6 and A11. While insulin is synthesized and stored in the pancreas as a hexamer, the hormonally active form is the monomer. Because the hormone contains many functional different groups, it is a good candidate to test our catalytic system for bioconjugation.

Coupling was first attempted in the pH range 7.4 using ethyl diazoacetate and TSPPFeCl as catalyst. Cobaltocene was used in the insertion of insulin to reduce iron(III) to iron(II). In the previous experiments, ligation of the amino group of the amino acid derivatives to iron is supposed to facilitate the reaction [17]. This is unlikely with insulin. In these experimental conditions, reaction of ethyl diazoacetate with insulin in PBS/acetonitrile (7/4) at 25 °c for 1 h afforded the coupling product with 90% insulin conversion containing 10% of double insertion (see Fig. 4 and Table 5). Purification was carried out by dialysis to remove reagents of molecular weight <3000 Da followed by HPLC chromatography to remove residual unconjugated insulin.

Table 5
Alkylation of N-terminus of insulin by TSPPFeCl-catalyzed carbenoid transfer reactions.

	Catalyst (conc.)	Diazo[a]	Conversion (%)	Mono/bisinserted ratio
1	TSPPRuCO (0.17 mM)	EDA (1 mM)	65	60:5
2	TSPPRuCO (0.034 mM)	EDA (0.25 mM)	14	99:1
3[b]	TSPPFeCl (0.034 mM)	EDA (0.25 mM)	90	90:10
4[b]	TSPPFeCl (0.034 mM)	S (0.25 mM)	Traces	
5[b]	TSPPFeCl (0.034 mM)	F (0.25 mM)	75	98:2
6[b]	TSPPFeCl (0.034 mM)	DDMP (0.25 mM)	Traces	
7[b]	TSPPFeCl (0.17 mM)	DDMP (0.25 mM)	20	100:0

Reaction conditions: the solution of insulin, diazo, and catalyst was stirred for 1 h at 25 °C in a PBS (pH 7.4)/MeCN (7/3) mixture.
[a] EDA = ethyl diazoacetate, F = 4-(trifluoromethyl)benzyl diazoacetate and S = 4-(thiomethyl)benzyl diazoacetate, DDMP = diisopropyl diazomethylphosphonate.
[b] one equiv of cobaltocene (vs catalyst) was added.



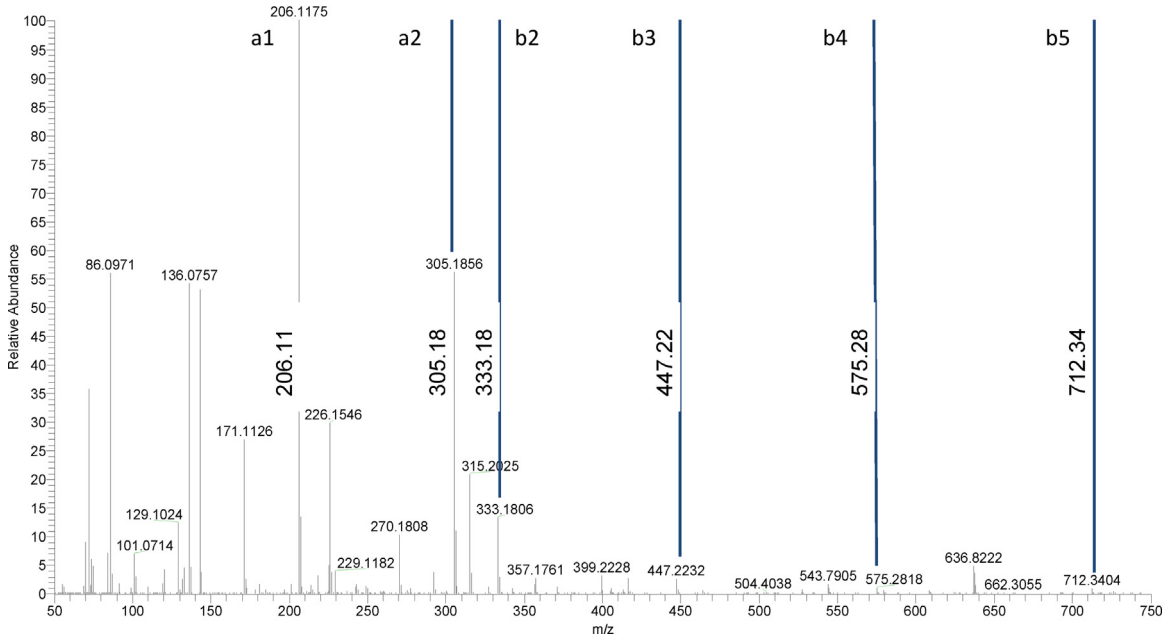


Fig. 5. Mass spectrum of insulin after ethyl diazoacetate coupling.

Analysis by MALDI-TOF MS upon purification by HPLC, revealed a peak at 5734 + 86 = 5820 (MH^+), consistent with the incorporation of one acetate carbene unit. Electrospray MS/MS (see experimental section and Fig. 5) of this mono-ligated species as compared to unmodified insulin, revealed attachment of the ester moiety to the FVNQHL peptide only, corresponding to the N termini of chain B. Fragmentation of this peptide yielded peaks at m/z = 206 (ion a_1), m/z = 305 (ion a_2), m/z = 333 (ion b_2), m/z = 447 (ion b_3), m/z = 676 (ion b_4) and m/z = 712 (ion b_5), according to the modification of the phenylalanine in *N*-termini. In contrast, fragmentation of chain A, revealed two unmodified peptides, one from the *N*-termini (GIVEQ) and the second to the acid termini (GERGFFYTPKA). This result showed that neither N termini nor the NH_2 of lysine was tethered by the ester group of the diazo precursor.

In order to confirm this result, ^{1}H NMR analysis of modified insulin revealed an AB spectrum for the CH_2 group, in alpha position of NH, located at 3.4 ppm as expected for a chiral amino acid. In contrast the CH_2 group ligated to Gly, revealed a singlet according to the results obtained with the amino ester models. Moreover, the methyl group, of the ligated acetate can also be detected in the NMR spectrum (Fig. 6).

Other diazo derivatives were also tested for coupling to insulin (see Table 5). Whereas 4-(methylsulfide)benzyl 2-diazoacetate failed to give any adduct, the coupling of 4-(trifluoromethyl)benzyl diazoacetate to insulin was quite successful with 75% conversion and a very small amount of bis-insertion (2%) as detected by MALDI (see Fig. 4B). A stoichiometric amount of

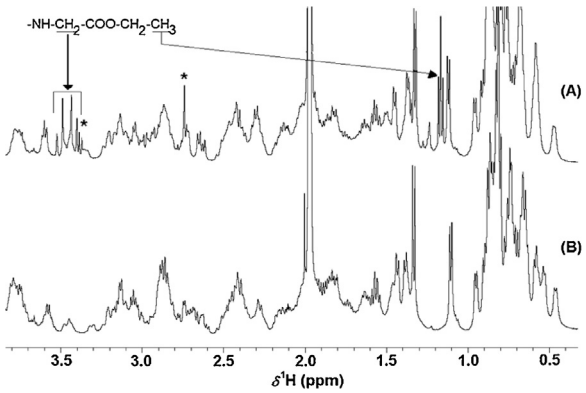


Fig. 6. ^{1}H NMR of insulin after (A) diazoacetate insertion and, (B) unmodified insulin.

TSPPFeCl was necessary to give 20% conversion with diisopropyl diazomethylphosphonate.

4. Conclusion

This paper suggests that diazo chemistry is not the perfect conjugation and assembly technology for all applications, but provides a powerful, attractive alternative to conventional chemistry. As a method for applying metal-catalyzed carbenoid transfer reactions for selective modification of proteins, various amino esters were

used as models for NH insertion reactions by four different diazo acetates using hydrosoluble porphyrin complex, TSPPFeCl. We conclude the absence of any O—H insertion, neither from methanol nor from water. It also shows a total regioselectivity (tryptophan and tyrosine). As observed with insulin, insertion is regioselective onto the NH_2 termini. In contrast, no regioselectivity was noted with cysteine. The introduction of new functional groups on specific sites containing NH groups can modify the stability of the macromolecules, their biological activity and the interactions between them. In the future, the challenge is to optimize all the possible parameters in water and in protic solvents using diazo ester compounds having functions adapted to the desired effects of the bioconjugation, and using catalysts which tolerate the different functions present on the protein.

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