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Protoporphyrin IX/Cobyrrinate Derived Hybrids – Novel Activators of Soluble Guanylyl Cyclase

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Abstract

A new cobyrrinate/protoporphyrin IX molecular hybrids were prepared via CuAAC reaction. The synthesis involved selective preparation of cobyrrinate and PpIX derived building blocks possessing respectively terminal alkyne and azide moieties followed by the CuOAc catalyzed cycloaddition reaction. Synthesized molecules activated soluble guanylyl cyclase showing strong linker length/activation dependence.

Keywords

Vitamin B₁₂; Protoporphyrin IX; enzyme activation; ‘click’ chemistry

Introduction

Hybrid molecules are defined as chemical entities having two or more structural motifs possessing different functions.^[1] In the field of medicinal chemistry, hybrid molecules (or multiple ligands)^[2] consist of at least two pharmacophores having either different or the same biological function designed to interact with one or more biological targets. Morphy classified this kind of molecules into (i) conjugated, where active sites are separated in space with a linker (ii) fused, when the length of the linker decreases, so the pharmacophores are close together and (iii) merged, when structural motifs of pharmacophores are mixed together in one, relatively low weight molecular framework.^[2] Such an approach towards drug design shows several advantages comparing to “drug cocktail”, namely shift of most optimization research (design and synthesis) to the earlier stage of drug discovery, lower risk of drug-drug interaction, more predictable pharmacokinetics etc.

Last decade witnessed a large interest in nitric oxide (NO) independent activators of soluble guanylyl cyclase (sGC),^[3] an enzyme that plays a crucial role in cardiovascular homeostasis, platelet function, angiogenesis and neurotransmission.^[4] The full structure of sGC is still unknown, but it is clear that this is a heterodimer with head-to-tail oriented subunits consisting of regulatory, catalytic domains and dimerization region. Regulatory domain possesses a prosthetic heme moiety which is crucial for NO sensing, while catalytic domain is responsible for the guanosine triphosphate (GTP) binding. This enzyme is

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activated by binding NO to the heme moiety, which triggers the upregulation of the conversion rate of GTP to cyclic guanosine monophosphate (cGMP) in catalytic domain. Activation of sGC is used in therapy by treating patients with NO releasing drugs such as organic nitrates, though a rapid tolerance is induced.^[4] With increasing doses of NO releasing substances other proteins can be affected, resulting in decreased drug selectivity and side effects. Therefore a search for new, NO independent sGC activators is of particular interest. Only a few NO independent regulators of sGC were reported.^[5] Although not all the details of mechanisms of their action are understood, it is clear that their function is dependent on the heme containing domain. Heme removal or change in the oxidation state of Fe²⁺ alters their activation properties. Among NO independent sGC activators of various structures,^[3c,5] protoporphyrin IX (PpIX, Figure 1) was found to be a very strong *in vitro* sGC activator,^[6] but experiments *in vivo* failed due to poor bioavailability.

Recently, we have reported, that dicyanocobinamide ((CN)₂Cbi, Figure 1) a vitamin B₁₂ derivative, is a novel, NO independent, sGC activator. Moreover, we have proven, that it targets directly the catalytic domain of sGC, responsible for GTP to cGMP conversion.^[7]

Since PpIX and (CN)₂Cbi are strong sGC activators targeting different domains, we envisaged, that a hybrid molecule containing PpIX and (CN)₂Cbi derived moieties connected with flexible polyglycol linker would show high, NO independent activation of sGC due to a combined synergistic effect.

In this article, we disclose a facile method for the synthesis of a hydrophobic (CN)₂Cbi derivative/PpIX hybrid molecule *via* copper catalyzed azide-alkyne cycloaddition (CuAAC, click methodology). In our study, we used hydrophobic heptamethylcobyrinate as a model compound. Several selective modifications of such hydrophobic derivatives have already been described,^[8] however, to the best of our knowledge, copper catalyzed azide-alkyne cycloaddition has not been explored for this purpose, though it was found to have practical applications in medicinal, bioconjugate, supramolecular chemistry and material science.^[9] Moreover, the use of CuAAC for connection of tetrapyrrole compounds is still challenging from the synthetic point of view.^[9e] We also report that the hybrid molecules are more potent sGC activators, than the individual building block of which they are composed.

Results and Discussion

The synthesis of a PpIX/cobyrrinate conjugate *via* CuAAC required preparation of 'clickable' building blocks. We had designed a hexamethyl ester derivative possessing an alkyne moiety and a PpIX with terminal azide. Vitamin B₁₂ derived *c*-acid **1**, obtained according to the procedure described by Kesse *et al.*^[10] was esterified with alkyne terminated alcohols using EDC/DMAP coupling conditions (Scheme 1). The reaction gave desired esters **2** and **3** in 91% and 89% yield respectively.

Following a similar procedure, PpIX was treated with readily synthesized linkers **4** and **5**^[11] affording monoesters **6** and **7** in good yield (Scheme 2). These monofunctionalized PpIX derivatives have a potential for future selective coupling reactions and other applications. Despite of our efforts, porphyrins **6** and **7** were obtained as a mixture of regioisomers, which we were not able to separate due lack of difference in polarity.

Initially, for the CuAAC, we investigated the conditions proposed originally by Sharpless and Fokin,^[13,14] which were used for the preparation of highly functionalized porphyrinoids,^[9e,12] including PpIX derivatives.^[15] The reaction of alkyne **2** with azide **6** in the presence of 30% CuSO₄/sodium ascorbate in *t*BuOH/water system^[13] afforded a complex mixture of products accompanied by starting materials (Table 1, entry 1).

ESIMS analysis of the crude reaction mixture showed that desired product **8** was formed, the peak observed at $m/z = 1761.6$ corresponded to the mass of $[M-CN]^{+}$. The presence of an additional peak at $m/z = 1822.7$ revealed that Cu insertion into the porphyrin core had occurred. Therefore, different reaction conditions were investigated. To the best of our knowledge, there are only two examples of CuAAC with porphyrin compounds in which copper insertion into the macrocyclic core is avoided by using *tris*-(benzyltriazolylmethyl)amine (TBTA).^[14] Unfortunately, in our case copper insertion was observed, even when excess TBTA was used in respect to the copper salt (entries 2 and 3). Therefore, by porphyrin **6** reacting with $Zn(AcO)_2 \cdot 2H_2O$ zinc protected porphyrin **Zn6** was obtained almost quantitatively. Due to the instability of porphyrin **Zn6** a brief work-up was conducted without chromatographic purification.

With protected porphyrin **Zn6** in hand, the search for an optimal catalytic system continued. When porphyrin **Zn6** was incorporated into a classical $CuSO_4/Na$ ascorbate or $CuI/TBTA$ system, a low yielding product was obtained with no improvement being detected upon solvent change or extended reaction time (entries 4–7). In recent literature one can find reports on highly tuned Cu(I) complexes that can accelerate CuAAC leading to full consumption of starting material within minutes.^[16] In addition, the majority of these systems were active in a very broad spectrum of solvents. Using a stable, well defined Cu(I) *N*-heterocyclic carbene complex introduced by Nolan^[16a] as extremely active CuAAC catalysts did not improve the conditions, as no trace of product was detected (entries 8 and 9). Similar results were obtained when using phenantroline based catalytic complex reported by Chen (entry 10).^[16b]

In 2010, Wang and Hu showed that $CuOAc$ could accelerate model Huisgen [1,3]-dipolar cycloaddition and decrease the reaction time to as short as 8 minutes.^[16c] Utilizing this method in DCM proved to be most successful, giving hybrid **Zn8** in 78 % yield in only 45 min. (entry 11). Contributing to the ease of this reaction was the straightforward purification process, making this method ideal for hybrid synthesis. Unfortunately the use of $CuOAc$ did not exclude Cu insertion (entry 12). Any further modifications to the reaction conditions, including changing solvent, temperature and reaction concentration, did not improve the yield significantly (entries 13–19).

Zn8 hybrid molecule (mixture of two regioisomers) was structurally characterized by 1H NMR, ^{13}C NMR, ESIMS, elemental analysis and UV/Vis spectroscopy. The 1H NMR spectra showed all characteristic signals for both the cobinamide and PpIX derivatives and signals at 7.87 and 7.86 derived from triazole moiety (Figure 2). The signal appears as two singlets because the obtained hybrid is a mixture of two regioisomers, which we were not able to separate using the applied chromatographic techniques.

Subsequent zinc removal from hybrid **Zn8** in DCM/TFA (3:1, reflux) led to free base compound **8** in 88% yield. Since the CuAAC step as well as Zn removal was conducted in the same solvent (dry DCM) a one-pot two-step procedure was explored (CuAAC + Zn removal). The CuAAC reaction of alkyne **2** with azide **Zn6** was followed by the addition of TFA and refluxing for 4 hours. This simple procedure furnished the best result and desired zinc free hybrid **8** was isolated in 70% yield with just one purification step.

To explore the effect of the linker length on sGC activation, hybrids **9** and **10** possessing longer chain linkers (12 in hybrid **9**, 14 in **10** versus 10 bonds in hybrid **8**, Figure 3) were prepared in a similar manner, starting from alkyne **3** and azides **Zn6** or **Zn7**, respectively (in with a 47% and 86% yield as a mixture of regioisomers).

The successful cooperation of pharmacophores included within one molecule is possible when neither active site obstructs the other. The absorption spectrum of hybrid molecule **8** in DCM is virtually the superposition of the spectra of individual chromophores **2** and **6**, indicating the lack of strong interaction between chromophores in the ground state (Figure 4).

Next, we evaluated the effect of newly generated hybrid compounds on sGC activity. The cGMP-forming activity of sGC was changed in the presence of hybrid compounds **8**, **9**, and **10** at 20 and 200 μ M concentrations (Figure 4). Dose-dependence studies demonstrated that sGC activation was close to saturation at 200 μ M (data not shown). sGC activation by these compounds was compared with activation by the lead compounds, e.g. protoporphyrin IX and (CN)₂Cbi, and by the building blocks that were used to generate the hybrids. Consistent with our previous reports, we observed a dose-dependent activation of sGC by dicyanocobinamide.^[7] As expected from past work, activation by PpIX was much stronger than any of the building blocks, and was already saturating at 2 μ M. The constituent porphyrin components of the tested hybrids (Figure 4, building blocks) showed some sGC activation, but the extent of activation was dependent on the nature of the constituent. E.g., the porphyrin component **6**, which carries the ester instead of the acid moiety, was less effective than the lead activator protoporphyrin IX. These results are consistent with previous finding that acid moieties of the porphyrin are crucial for the proper interaction with the heme domain of sGC.^[18]

At the same time, both cobyrinate components (compounds **2** and **3**), showed the same potency as the lead activator dicyanocobinamide.

Although the activation of sGC by hybrid **8** was stronger than its respective building blocks, its activating potency is almost equal to the activity of both building blocks e.g. cobyrinate and porphyrin derivatives, combined. Compound **10** displayed moderate sGC activation, while hybrid **9**, in which both subunits are 12 bonds apart, was the most effective. Moreover, compound **9** activated sGC significantly stronger than any of the constituting components by themselves. This synergistic effect strongly suggests that the length of the linker connecting the porphyrin and cobyrinate moieties plays an important role in determining the efficiency of sGC activation.

Conclusions

In summary, the deliberate and rational design of hybrids that target two sGC domains: regulatory and catalytic was described. Protoporphyrin IX was successfully coupled to hydrophobic vitamin B₁₂ derivative via [1,3]-dipolar cycloaddition. The results proved CuOAc being the best catalyst for this reaction giving desired hybrid molecule **8** in 75% yield. To the best of our knowledge, this is the first example of vitamin B₁₂ derivative being used in CuAAC.

Biological results revealed that hybrids **9** and **10** activated sGC significantly stronger than any of the constituting components by themselves, confirming the beneficial effect of combining two sGC activators in one molecule. Moreover, it has been found that the length of the chain linker had a profound effect on the extent of sGC activation. The maximal fold of activation was observed for compound **9**, in which both activators are separated by 12 bonds. Future studies are required to determine the optimal length and composition of the linker to provide the most efficient activation of sGC.

Experimental Section

General Information

Solvents for chromatography (pure grade) were distilled prior to use. Vitamin B₁₂, protoporphyrin IX and CuOAc were purchased from Sigma-Aldrich, and used as received. DCVC^[19] (dry column vacuum chromatography) was performed using Kiesgel 60G (13% of plaster). Flash chromatography was performed using Kiesgel 60 (0.08 mm, 200 mesh). Thin layer chromatography was performed using aluminium plates covered with Silica Gel GF₂₅₄, 0.20 mm thickness. ¹H and ¹³C NMR spectra were recorded at r.t. on 400 or 500 or 600 MHz spectrometers with TMS or residual solvent peak as an internal standard.

Synthesis of alkyne 2

Monoacid **1**^[10] (270 mg, 0.23 mmol) was dissolved in dry DCM (25 mL) under argon and then the deep orange solution was cooled to 0 °C. EDC (130 mg, 0.70 mmol) and DMAP (85 mg, 0.70 mmol) were then added in one portion, followed by the addition of propargyl alcohol (120 µL, 2.0 mmol). The reaction mixture was allowed to warm to room temperature and then stirred overnight. It was then diluted with DCM and washed with phosphate buffer (pH = 7) containing approx. 1 % of KCN. The violet coloured organic phase was then dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified using DCVC (5% EtOH in DCM). The isolated pure product was redissolved in DCM and washed with phosphate buffer (pH = 7) containing approx. 1% of KCN. The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. Precipitation from AcOEt/hexane gave alkyne **2** as violet crystals (240 mg, 91%). *R*_f = 0.45 (5% EtOH in DCM); Elemental analysis calcd. (%) for C₅₆H₇₃CoN₆O₁₄ + H₂O: C 59.46, H 6.68, N 7.43; found: C 59.59, H 6.94, N 7.34; HRMS (ESI): *m/z* calcd. for: C₅₅H₇₃CoN₅O₁₄ [M-CN⁺]: 1086.4481; found: 1086.4533; UV/Vis (DCM): λ (ε L·mol⁻¹·cm⁻¹) = 589.3 (1.09×10⁴), 549.6 (8.51×10³), 422 (2.86×10³), 371 (2.82×10⁴), 316.5 (9.25×10³), 278.8 (1.2×10⁴) nm; ¹H NMR (500 MHz, toluene-d₈): δ = 5.67 (bs, 1H, CH *meso*), 4.42 (dd, *J* = 1.9 Hz, *J* = 15.5 Hz, 1H, O-CH₂-C≡CH), 4.25 (dd, *J* = 1.9 Hz, *J* = 15.5 Hz, 1H O-CH₂-C≡CH), 3.94 (d, *J* = 10.4 Hz, 1H), 3.90-3.85 (m, 1H), 3.61-3.54 (m, 1H), 3.47 (s, 3H, O-CH₃), 3.39 (s, 3H O-CH₃), 3.38 (s, 6H, 2× O-CH₃), 3.33 (s, 3H, O-CH₃), 3.29 (s, 3H, O-CH₃), 2.90-2.79 (m, 3H), 2.72-2.43 (m, 8H), 2.42-2.33 (m, 3H), 2.32-2.21 (m, 4H), 2.20 (s, 3H, CH₃), 2.04 (s, 3H, CH₃), 2.00-1.91 (m, 4H), 1.84-1.65 (m, 3H), 1.44 (s, 3H, CH₃), 1.36 (s, 3H, CH₃), 1.69 (s, 3H, CH₃), 1.12 (s, 3H, CH₃), 0.95 (s, 6H, 2×CH₃) ppm; ¹³C NMR (125 MHz, toluene-d₈): δ = 176.1, 174.9, 173.3, 172.7, 172.3, 172.0, 171.7, 171.3, 171.2, 169.1, 163.3, 91.0, 82.5, 77.9, 74.8, 74.5, 58.1, 56.7, 54.1, 53.6, 51.3, 51.2, 50.9, 50.8, 50.6, 50.5, 48.7, 46.5, 45.7, 41.9, 41.2, 39.4, 34.6, 33.4, 32.7, 31.5, 30.9, 30.3, 30.1, 29.5, 26.9, 26.5, 25.5, 25.3, 24.6, 22.7, 22.5, 21.9, 19.0, 18.8, 17.7, 16.6, 15.5, 15.1, 13.9, 11.2 ppm.

Synthesis of alkyne 3

Alkyne **3** was obtained following the similar procedure from acid **1** and 4-pentyn-1-ol in 89% yield. Violet crystals *R*_f=0.45 (5% EtOH in DCM); Elemental analysis calcd. (%) for C₅₈H₇₇CoN₆O₁₄: C 61.04, H 6.80, N 7.36; found: C 60.90, H 6.84, N 7.16; MS (ESI): *m/z* calcd. for C₅₇H₇₇CoN₅O₁₄ [M-CN⁺]: 1114.5; found: 1114.4; UV/Vis (DCM): λ (ε L·mol⁻¹·cm⁻¹) = 589.3 (0.93×10⁴), 550.6 (7.31×10³), 422 (2.36×10³), 371 (2.45×10⁴), 315.0 (7.86×10³), 278.8 (9.93×10³) nm; ¹H NMR (500 MHz, toluene-d₈): δ = 5.68 (bs, 1H), 3.94 (t, *J* = 6.3 Hz, 3H), 3.89 (t, *J* = 5.5 Hz, 1H), 3.63 (dd, *J* = 5.0 Hz, *J* = 3.5 Hz, 1H), 3.47 (s, 3H), 3.403 (s, 3H), 3.339 (s, 3H), 3.39 (s, 3H), 3.34 (s, 3H), 3.29 (s, 3H), , 2.88-2.81 (m, 4H), 2.71-2.54 (m, 4H), 2.54-2.46 (m, 4H), 2.46-2.23 (m, 8H), 2.21 (s, 3H), 2.04 (s, 3H) 1.98-1.94 (m, 4H), 1.79 (t, *J* = 2.5 Hz, 1H), 1.53-1.47 (m, 3H), 1.45 (s, 3H), 1.41 (s, 3H), 1.21 (bs, 1H), 1.18 (s, 3H), 1.15 (s, 3H), 1.00 (s, 3H), 0.99 (s, 3H) ppm; ¹³C NMR (125 MHz, toluene-d₈): δ = 176.0, 175.1, 174.8, 173.4, 172.8, 172.8, 172.3, 172.0, 171.7, 171.4,

171.3, 169.9, 163.6, 163.1, 103.3, 102.3, 91.1, 82.9, 82.5, 74.8, 69.2, 58.1, 56.7, 54.2, 53.7, 51.3, 50.9, 50.9, 50.6, 50.5, 48.8, 46.5, 45.7, 42.1, 41.2, 39.4, 34.6, 33.4, 32.7, 31.6, 31.5, 30.8, 30.4, 30.1, 29.5, 29.1, 27.3, 26.9, 26.4, 25.5, 25.2, 24.6, 22.7, 21.9, 19.1, 18.9, 17.7, 16.5, 15.1, 15.0, 13.9, 11.2 ppm.

Synthesis of porphyrin 6

Protoporphyrin IX (150 mg, 0.26 mmol) was dissolved in dry DMF (20 mL) under argon and the resulting solution was cooled to 0 °C. EDC (52 mg, 0.27 mmol) and DMAP (33 mg, 0.27 mmol) were then added, followed by the addition of alcohol **4** (35 mg, 0.27 mmol) in DMF (2 mL). The reaction mixture was allowed to warm to room temperature and then stirred overnight. It was then diluted with a solution of NH₄Cl and extracted with DCM. The organic phase was washed with water and brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified using DCVC (gradually from 2 to 7% MeOH in DCM), the second most intensive band was collected and evaporated to dryness. After precipitation from DCM/hexane; porphyrin **6** was obtained as a brown solid (106 mg, 61%). *R_f* = 0.4 (5% MeOH in DCM); Elemental analysis. calcd. (%) for C₃₈H₄₁N₇O₅ + H₂O C 65.78, H 6.25, N 14.13; found: C 65.75, H 6.14, N 14.10; HRMS (ESI): *m/z* calcd. for: C₃₈H₄₂N₇O₅ [M+H⁺] 676.3242; found: 676.3224; UV/Vis (DCM): λ (ε L·mol⁻¹·cm⁻¹) = 669 (2.3×10³), 693 (4.69×10³), 576 (7.30×10³), 540 (1.04×10⁴), 505 (1.20×10⁴), 406 (1.59×10⁵) nm; ¹H NMR (400 MHz, CDCl₃, TMS): δ = 9.80 and 9.79 (s,s, 1H, CH *meso*), 9.73 and 9.72 (s,s, 1H CH *meso*), 9.65 and 9.64 (s, s, 1H, CH *meso*), 9.62 (s, 1H, CH *meso*), 8.15-7.93 (m, 2H, vinyl), 6.30-6.03 (m, 4H, vinyl), 4.25-4.04 (m, 6H), 3.47 (s, 3H, -CH₃), 3.45 and 3.44 (s,s, 3H, -CH₃), 3.43 and 3.41 (s,s, 3H, -CH₃), 3.39 and 3.37 (s,s, 3H, -CH₃), 3.36-3.30 (m, 2H), 3.19-3.09 (m, 4H), 3.03 (t, *J* = 4.7 Hz, 2H), 2.76 (t, *J* = 4.7 Hz, 2H), -4.50 ppm (bs, 2H, NH pyrrole); ¹³C NMR (100 MHz, CDCl₃, TMS): δ = 176.5, 173.6, 130.1, 130.0, 129.9, 120.2, 120.1, 97.2, 96.7, 96.4, 96.6, 69.3, 68.8, 63.4, 49.9, 36.9, 36.6, 21.6, 21.3, 12.0, 11.4, 11.3 ppm.

Synthesis of porphyrin 7

Porphyrin **7** was prepared using similar procedure from PpIX and alcohol **5** in 58% yield. Brown solid, *R_f* = 0.4 (5% MeOH in DCM), Elemental analysis. calcd. (%) for C₄₀H₄₅N₇O₆: C 66.74, H 6.30, N 13.62; found: C 66.56, H 6.40, N 13.23; HRMS (ESI): *m/z* calcd. for: C₄₀H₄₆N₇O₆ [M+H⁺] 720.35041; found: 720.3498; UV/Vis (DCM): λ (ε L·mol⁻¹·cm⁻¹) = 669 (2.32×10³), 693 (4.77 ×10³), 576 (7.30 ×10³), 540 (1.08×10⁴), 504 (1.20×10⁴), 406 (1.60×10⁵) nm; ¹H NMR (500 MHz, CDCl₃, TMS): δ = 9.62 and 9.61 (s, s, 1H), 9.48 and 9.47 (s, s, 1H), 9.45 and 9.44 (s, s, 1H), 9.40 and 9.38 (s, s, 1H), 8.11-7.76 (m, 2H), 6.30-5.97 (m, 4H), 4.25-4.16 (m, 2H), 4.14-3.95 (m, 4H), 3.47-3.40 (m, 2H), 3.37 (s, 3H), 3.36 and 3.35 (s, s, 3H), 3.34 and 3.33 (s, s, 3H), 3.30 and 3.27 (s, s, 3H), 3.16-3.05 (m, 6H), 3.33-2.97 (m, 2H), 2.96-2.90 (m, 2H), 2.89-2.83 (m, 2H), -4.50 (bs, 2H) ppm; ¹³C NMR (125 Hz, CDCl₃, TMS): δ = 176.3, 173.8, 130.4, 120.4, 97.4, 96.9, 96.6, 95.8, 70.1, 70.04, 69.6, 69.2, 63.7, 50.4, 37.2, 37.1, 21.9, 12.7, 11.6 ppm.

Synthesis of porphyrin Zn6

Porphyrin **6** (17 mg, 0.025 mmol) was dissolved in DCM/MeOH (1:1, 20 mL) and a large excess of Zn(AcO)₂·2H₂O (170 mg, 0.75 mmol) was added. The mixture was stirred at room temperature for 2 hours. It was then diluted with DCM and washed with a saturated solution of NH₄Cl and water. The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. Precipitation from DCM/hexane gave porphyrin **Zn6** as a dark purple solid (19 mg). It was used for the next steps without further purification. MS (ESI): *m/z* calcd. for: C₃₈H₃₉N₇O₅Zn + Na⁺ [M+Na⁺] 760.22; found: 760.30, UV/Vis (DCM): λ = 653, 577, 540, 410, 355 nm.

Synthesis of porphyrin Zn7

Porphyrin **Zn7** was prepared using similar procedure starting from porphyrin **7**. It was used for the next steps without further purification. MS (ESI): m/z calcd. for: $C_{40}H_{43}N_7O_6Zn + Na^+$ [M+Na⁺] 804.3; found: 804.3.

Optimization of the 'click reaction' between 2 and 6 or Zn6 – general procedure

Porphyrin **6** (9 mg, 0.02 mmol) or **Zn6** (10 mg, 0.02 mmol) and alkyne **2** (17 mg, 0.02 mmol) were dissolved under argon in the respective degassed solvent (3 mL) and a copper catalyst (0.005 mmol) was added followed by the respective additive (reducing agent or ligand). All reactions were monitored by TLC. The reactions were quenched using phosphate buffer (pH = 7) containing approx. 1% of KCN and extracted with DCM. The combined organic layers were then dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified using flash column chromatography (gradually from 1 to 7% MeOH in DCM). Isolated product was redissolved in DCM and washed with phosphate buffer (pH = 7) containing approx. 1% of KCN, dried over Na_2SO_4 , filtered and concentrated *in vacuo*.

Synthesis of hybrid Zn8

Porphyrin **Zn6** (19 mg, 0.03 mmol) and alkyne **2** (28 mg, 0.03 mmol) were placed into a Schlenk tube, which was flushed with argon before the addition of dry DCM (12 mL). $CuOAc$ (1 mg, 0.008 mmol) was added and the mixture was vigorously stirred. After 45 minutes the reaction was quenched with phosphate buffer (pH = 7, 100 mL) containing approx. 1% of KCN and the organic phase as dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified using flash column chromatography (from 1 to 7% MeOH in DCM). The isolated product was redissolved in DCM and washed with phosphate buffer containing approx. 1% of KCN. The organic phase was dried over Na_2SO_4 , filtered and concentrated *in vacuo*. After precipitation from DCM/hexane hybrid **Zn8** was obtained as a dark violet solid (37 mg, 75%). R_f = 0.3 (5% MeOH in DCM); Elemental analysis calcd. (%) for $C_{94}H_{112}CoN_{13}O_{19}Zn$: C 60.95, H 6.09, N 9.83; found: C 61.01, H 6.19, N 9.50; MS(ESI); m/z calcd. for: $C_{93}H_{112}CoN_{12}O_{19}Zn$ [M-CN⁺] 1823.7; found: 1823.8; calcd. for: $C_{93}H_{111}CoN_{12}O_{19}Zn + Na^+$ [M-HCN+Na⁺] 1845.7; found: 1845.8; calcd. for: $C_{94}H_{112}CoN_{13}NaO_{19}Zn$ [M+Na⁺] 1872.7; found: 1872.8; $C_{94}H_{111}CoN_{13}O_{19}Zn + 2Na^+$; calcd. for: [M-H+2Na⁺] 1894.7; found: 1894.8; UV/Vis (DCM): λ (ϵ L·mol⁻¹·cm⁻¹) = 584 (1.93×10^4), 548 (1.91×10^4), 421 (1.44×10^5), 370 (4.51×10^4), 277 (1.97×10^4) nm; ¹H NMR (600 MHz, DMSO- d_6): δ = 12.25 (bs, 1H, COOH), 10.26 (s, 1H, CH *meso* PpIX), 10.18 (s, 1H, CH *meso* PpIX), 10.16 (s, 1H, CH *meso* PpIX), 10.10 (s, 1H, CH *meso* PpIX), 8.57-8.45 (m, 2H, vinyl), 7.87, 7.86 (s, s, 1H, CH triazole), 6.45-6.34 (m, 2H, vinyl), 6.18-6.07 (m, 2H, vinyl), 5.55 (s, 1H, CH *meso* vit B₁₂), 5.05 (s, 2H, , CH₂-CO₂-CH₂-C_{triazole}), 4.39-4.28 (m, 4H, 2 × C_{PpIX}-CH₂-CH₂), 4.26-4.20 (m, 2H, N_{triazole}-CH₂), 4.08 (bs, 2H), 3.78-3.70 (m, 6H), 3.66 (s, 3H), 3.62 (s, 3H), 3.64-3.45 (m, 6H), 3.60 (s, 6H), 3.53 (s, 3H), 3.52 (s, 3H), 3.47-3.37 (m, 3H), 3.28 (t, J = 6 Hz, 2H), 3.20-3.12 (m, 3H), 2.90-2.85 (m, 1H), 2.82-2.70 (m, 2H, CH₂-CO₂-CH₂-C_{triazole}), 2.67-2.58 (m, 3H), 2.47-2.35 (m, 3H), 2.34-2.25 (m, 3H), 2.24-2.07 (m, 5H), 2.05 (s, 3H, *meso*-CH₃), 2.02 (s, 3H *meso*-CH₃), 1.99-1.68 (m, 6H), 1.63-1.46 (m, 4H) 1.45 (s, 3H, CH₃), 1.29 (s, 3H, CH₃), 1.25 (s, 3H, CH₃), 1.18 (s, 3H, CH₃), 1.11 (s, 3H, CH₃), 0.91 ppm (s, 3H, CH₃); ¹³C NMR (150 MHz, DMSO- d_6): δ = 176.0, 175.4, 175.3, 173.7, 173.3, 173.0, 172.8, 172.75, 172.74, 172.0, 171.0, 170.1, 163.2, 162.9, 148.2, 148.1, 147.9, 147.8, 147.5, 147.2, 146.5, 146.0, 141.4 (C_{triazole}), 139.7, 139.2, 137.3, 137.2, 136.8, 136.6, 136.5, 130.9 (vinyl), 130.8 (vinyl), 124.8 (C-H_{triazole}), 119.5 (vinyl), 103.2 (*meso* Vit B₁₂), 101.6 (*meso* Vit B₁₂), 98.1 (*meso* PpIX), 97.7 (*meso* PpIX), 97.4 (*meso* PpIX), 96.9 (*meso* PpIX), 90.2 (C-H, *meso* Vit B₁₂), 82.1, 73.9, 68.3, 67.8, 63.1, 57.6, 57.3, 56.2, 53.3, 52.3, 51.9, 51.6, 51.5, 51.4, 49.0, 47.9,

46.1, 45.2, 41.6, 40.9, 37.2, 36.9, 32.9, 31.4, 30.9, 30.6, 30.5, 30.3, 30.2, 29.1, 26.3, 25.9, 25.2, 24.7, 24.2, 22.0, 21.6, 21.5, 21.4, 18.9, 18.7, 17.5, 16.1, 15.1, 14.7, 13.9, 12.8, 11.4 ppm.

Removal of Zn from hybrid Zn8 - synthesis of hybrid 8

Hybrid **Zn8** (22 mg, 0.01 mmol) was placed into a Schlenk tube, which was flushed with argon before the addition of dry DCM (6.0 mL) and TFA (2.0 mL). The reaction mixture was heated to reflux for 4 hours and then stirred overnight at room temperature. It was then diluted with DCM and poured into a solution of phosphate buffer (pH = 7). The organic phase was separated and washed with phosphate buffer containing KCN (5.0 mg), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified using flash column chromatography (from 1 to 7% MeOH in DCM). It was then redissolved in DCM and washed with phosphate buffer (pH = 7, 100 mL) containing approx. 1% of KCN. The organic phase was dried over Na₂SO₄, filtered and concentrated *in vacuo*. After precipitation from DCM/hexane hybrid **8** was obtained as a dark brownish solid (19 mg, 88%). *R*_f = 0.3 (5% MeOH in DCM); elemental analysis calcd. (%) for C₉₄H₁₁₄CoN₁₃O₁₉: C 63.11, H 6.42, N 10.18; found C 63.13, H 6.55, N 9.93; ESIMS *m/z* calcd. for C₉₃H₁₁₄CoN₁₂O₁₉ [M-CN]⁺ 1761.8; found 1761.8, calcd. for C₉₃H₁₁₃CoN₁₂O₁₉+Na⁺ [M-HCN+Na]⁺ 1783.7, found 1783.7; UV/Vis (DCM): λ (εL·mol⁻¹·cm⁻¹) = 667 (8.39×10²), 629 (3.66×10³), 579 (1.32×10⁴), 542 (1.57×10⁴), 508 (1.49×10⁴), 409 (1.31×10⁵), 278 (2.27×10⁴) nm; ¹H NMR (600 MHz, DMSO- *d*₆): δ = 12.27 (bs, 1H, COOH), 10.15 (bs, 1H, *meso* PpIX), 10.13 (m, 2H, *meso* PpIX), 10.09, 10.10 (s, s 1H, *meso* PpIX), 8.50-8.35 (m, 2H, vinyl), 7.80, 7.97 (s, s, 1H, triazole), 6.61 (bd, *J* = 17 Hz, 2H, vinyl), 6.20 (bd, *J* = 17 Hz, 2H, vinyl), 5.51 (s, 1H, *meso* vit B₁₂), 5.01 (s, 2H), 4.37-4.25 (m, 4H), 4.11 (dd, *J* = 5.5 Hz, *J* = 11.0 Hz, 2H), 4.08-4.03 (m, 2H), 3.75-3.69 (m, 2H), 3.67 (s, 3H, CH₃), 3.66 (s, 3H, CH₃), 3.69-3.64 (m, 2H), 3.62 (s, 3H, CH₃), 3.59 (s, 3H, CH₃), 3.58 (s, 3H, CH₃), 3.61-3.53 (m, 3H), 3.51 (s, 3H, CH₃), 3.50 (s, 3H, CH₃), 3.54-3.45 (m, 2H), 3.44-3.36 (m, 3H), 3.26 (t, *J* = 7.4 Hz, 2H), 3.16 (t, *J* = 7.4 Hz, 2H), 3.12 (t, *J* = 6.1 Hz, 1H), 2.85-2.71 (m, 3H), 2.65-2.54 (m, 3H), 2.46-2.34 (m, 3H), 2.33-2.17 (m, 5H), 2.19-1.90 (m, 3H), 2.03 (s, 3H, CH₃), 1.96 (s, 3H, CH₃), 1.90-1.78 (m, 3H), 1.76-1.64 (m, 3H), 1.64-1.53 (m, 1H), 1.50-1.42 (m, 3H), 1.40 (s, 3H, CH₃), 1.28 (s, 3H, CH₃), 1.24 (s, 3H, CH₃), 1.11 (s, 3H, CH₃), 1.09 (s, 3H, CH₃), 0.86 (s, 3H, CH₃), -4.20 ppm (bs, 2H, NH pyrrole); ¹³C NMR (150 MHz, DMSO- *d*₆) δ = 175.4, 174.9, 174.8, 174.0, 173.1, 172.8, 172.4, 172.3, 172.27, 172.25, 171.5, 170.5, 169.5, 162.7, 162.4, 141.4, 130.0, 124.8, 121.0, 103.2, 101.6, 97.6, 97.3, 97.0, 96.9, 90.2, 82.1, 74.0, 68.2, 67.8, 63.1, 57.6, 57.3, 56.2, 53.3, 51.9, 51.6, 51.4, 51.2, 48.9, 47.9, 46.0, 45.2, 41.6, 41.0, 38.8, 36.7, 36.4, 34.2, 32.9, 31.4, 30.9, 30.5, 30.3, 30.2, 29.1, 26.3, 25.9, 25.2, 24.2, 22.0, 21.6, 21.2, 21.1, 18.8, 18.7, 17.5, 16.1, 15.1, 14.7, 13.9, 12.6, 11.3 ppm.

One-pot synthesis of hybrid 8

Porphyrin **Zn6** (19 mg, 0.03 mmol) and alkyne **2** (28 mg, 0.03 mmol) were placed into a Schlenk tube, which was flushed with argon before the addition of dry DCM (12 mL). CuOAc (1.0 mg, 0.008 mmol) was added and the mixture was vigorously stirred. The reaction was monitored by TLC, when it was complete TFA (1 mL) was added and the mixture was refluxed for 4 hours, allowed to cool to room temperature. After standard workup, purification and precipitation (see above) hybrid **8** was obtained as a dark brownish solid (31 mg, 70%).

Synthesis of hybrid 9

Compound **9** was obtained following similar procedure from porphyrin **Zn6** and alkyne **3** in 47% yield. Dark brownish solid, *R*_f = 0.3 (5% MeOH in DCM); elemental analysis calcd. (%) for C₉₆H₁₁₈CoN₁₃O₁₉+ 2H₂O: C 62.22, H 6.64, N 9.83; found C 62.31, H 6.69, N 9.61;

ESIMS m/z calcd. for $C_{96}H_{118}CoN_{13}O_{29} [M-CN]^+$ 1789.8; found 1789.9, calcd. for $C_{96}H_{118}CoN_{13}O_{19}+Na^+ [M+Na]^+$ 1838.8, found 1838.9; UV/Vis (DCM): λ ($\text{eL}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) = 629 (4.31×10^3), 574 (1.17×10^4), 540 (1.72×10^4), 505 (1.74×10^4), 406 (1.45×10^5), 276 (2.16×10^4) nm; ^1H NMR (500 MHz, DMSO- d_6 , 85 °C): δ = 14.40 (bs, 1H), 10.18 (s, 1H), 10.15 (s, 1H), 10.14 (s, 1H), 10.12 (s, 1H), 8.40-8.31 (m, 2H), 7.36 (s, 1H), 6.45-6.36 (m, 2H), 6.24-6.17 (m, 2H), 5.58 (s, 1H), 4.40-4.30 (m, 4H), 4.13-4.08 (m, 2H), 4.07-4.02 (m, 2H), 4.00-3.93 (m, 2H), 3.73 (d, J = 10.0 Hz, 2H), 3.70 (s, 3H), 3.68 (s, 3H), 3.67, 3.66 (s,s, 3H), 3.65 (s, 3H), 3.63 (s, 3H), 3.62 (s, 3H) 3.61(s, 2H), 3.60 (s, 3H), 3.58 (s, 3H), 3.55 (s, 3H), 3.54 (s, 3H), 3.52-3.47 (m, 3H), 3.44-3.39 (m, 3H), 3.29 (t, J = 7. Hz, 3H), 3.24-3.17 (m, 3H), 2.92-2.86 (m, 2H), 2.78- 2.71 (m, 2H), 2.70-2.52 (m, 5H), 2.47-2.29 (m, 4H), 2.28-2.12 (m, 4H), 2.11 (s, 3H), 2.07 (m, 3H), 2.04-1.83 (m, 4H), 1.82-1.75(m, 2H), 1.47 (s, 3H), 1.37 (s, 3H), 1.31 (s, 3H), 1.21 (s, 3H), 1.14 (s, 3H), 0.98 (s, 3H), -3.86 (bs, 2H) ppm; ^{13}C NMR (125 MHz, DMSO- d_6 , 85 °C): δ = 175.1, 174.7, 174.5, 172.5, 172.2, 171.8, 171.74, 171.71, 171.5, 170.9, 170.3, 169.3, 162.5, 162.4, 162.3, 145.2, 143.9, 143.7, 136.7, 136.5, 135.9, 135.8, 129.64, 129.63, 129.61, 121.24, 120.51, 120.46, 102.8, 101.2, 97.2, 96.8, 96.6, 96.3, 89.8, 81.8, 73.9, 68.0, 67.6, 63.1, 62.7, 57.3, 56.1, 53.4, 52.4, 51.3, 50.96, 50.95, 50.8, 50.57, 50.55, 48.4, 47.7, 45.8, 45.3, 41.8, 40.8, 38.7, 36.3, 36.0, 31.4, 30.4, 30.3, 30.2, 28.8, 27.3, 25.5, 24.9, 24.0, 20.8, 18.49, 18.47, 17.0, 15.7, 14.6, 13.1, 11.8, 10.7 ppm;

Synthesis of hybrid 10

Compound **10** was obtained following similar procedure from porphyrin **Zn7** and alkyne **3** in 86% yield. Dark brownish solid, R_f = 0.3 (5% MeOH in DCM); elemental analysis calcd. (%) for $C_{98}H_{122}CoN_{13}O_{20}+ H_2O$: C 62.64, H 6.65, N 9.69; found C 62.50, H 6.74, N 9.44; ESIMS m/z calcd. for $C_{97}H_{122}CoN_{12}O_{20} [M-CN]^+$ 1833.8; found 1833.9, calcd. for $C_{98}H_{122}CoN_{13}O_{20}+Na^+ [M+Na]^+$: 1882.8, found 1882.9; UV/Vis (DCM): λ ($\text{eL}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) = 629 (3.79×10^3), 574 (1.37×10^4), 541 (1.59×10^4), 506 (1.42×10^4), 406 (1.34×10^5), 277 (2.09×10^4) nm; ^1H NMR (500 MHz, DMSO- d_6 , 85 °C) δ = 10.24 (s, 1H), 10.20 (s, 1H), 10.18 (s, 1H), 10.17 (s, 1H), 8.45-8.33 (m, 2H), 7.48 (s, 1H), 6.68-6.18 (m, 4H), 5.60 (m, 1H), 4.42-4.31 (m, 4H), 4.18-4.12 (m, 2H), 4.11-4.07 (m, 2H), 4.05-3.99 (m, 2H), 3.76-3.73 (m, 2H), 3.71 (s, 3H), 3.70 (s, 3H), 3.69 (s, 3H), 3.65 (s, 3H), 3.64 (s, 3H), 3.63 (s, 3H), 3.62 and 3.61 (s,s, 3H), 3.60 (s, 3H), 3.56 (s, 3H), 3.55 (s, 3H), 3.45-3.41 (m, 2H), 3.38-3.35 (m, 2H), 3.33-3.27 (m, 4H), 3.25-3.17 (m, 4H), 3.16-3.12 (m, 2H), 3.09-3.05 (m, 2H), 2.95-2.89 (m, 1H), 2.80-2.70 (m, 1H), 2.69-2.50 (m, 6H), 2.47-2.15 (m, 10H), 2.13 (s, 3H), 2.09 (s, 3H), 2.07-1.74 (m 5H), 1.68-1.54 (m, 2H), 1.50 (s, 3H), 1.37 (s, 3H), 1.31 (s, 3H), 1.24 (s, 3H), 1.15 (s, 3H), 1.00 (s, 3H), -4.50 (bs, 2H) ppm; ^{13}C NMR (125 MHz, DMSO- d_6 , 85 °C): δ = 175.1, 174.6, 174.4, 173.1, 172.5, 172.23, 171.9, 171.71, 171.69, 171.5, 170.9, 170.3, 169.3, 162.5, 162.3, 145.2, 136.5, 136.0, 129.6, 121.3, 120.5, 102.8, 101.2, 97.3, 96.8, 96.6, 96.3, 89.8, 81.8, 73.9, 68.9, 68.9, 68.0, 67.7, 63.1, 62.81, 57.4, 56.0, 53.4, 52.5, 51.3, 50.9, 50.8, 50.6, 50.5, 48.5, 47.7, 45.9, 45.3, 41.8, 40.8, 38.7, 36.2, 36.0, 32.7, 31.4, 30.4, 30.3, 30.2, 30.0, 28.8, 27.4, 25.5, 24.9, 24.0, 21.1, 21.0, 20.9, 20.8, 18.5, 17.0, 15.7, 14.7, 14.2, 11.8, 10.7 ppm.

Generation of recombinant human sGC enzyme

To generate recombinant sGC enzyme, the open reading frames of the α subunit and β subunits were cloned into the transfer vector pBacPak9 (Clontech, Mountain View, CA) to obtain the pBacPak- α and the pBacPak- β plasmids, respectively. A hexahistidine tag was also inserted at the C-terminus of the α subunit by PCR mutagenesis. Each of these plasmids were cotransfected with linearized baculovirus DNA (BaculoGold from Pharmingen, San Diego, CA) into Sf9 cells, according to the manufacturer's protocol, to generate baculoviruses expressing α or β sGC subunits. To express functional sGC, 5 liters of Sf9 cells were infected with both viruses and after 72 hours the cells were collected.

Recombinant sGC was purified from the lysates of these cells, as described previously (Martin et al., 2001). In short, 30 ml Ni-NTA-agarose column was loaded with the high-speed supernatant of Sf9 cell lysates, followed by extensive washes with 50 mM imidazole. Recombinant sGC (> 90% purity) was eluted by 200 mM imidazole pH 7.5, concentrated by ultrafiltration and subjected to gel-filtration for imidazole removal. The sample of purified sGC was supplied with 25 % glycerol and stored at -80°C .

sGC assay

Purified sGC (0.5 μg at 1 mg/ml) was added to reaction buffer (60 μl , triethanolamine (65 mM), EGTA (415 μM), albumin (0.5 mg/ml), cGMP (4 mM), creatine kinase (0.25 mg/ml), creatine phosphate (20 mM), and magnesium chloride (7.5 mM)) and was provided with hybrid compounds or their building blocks at indicated concentration. After 10 minutes incubation at room temperature, substrate buffer (40 μl , 500 μM GTP/0.08 μCi of [α - ^{32}P]GTP, ~ 150000 cpm) was added to initiate the cGMP formation and the sample was transferred to 37°C . After 10 minutes incubation, the reaction was stopped by sequential addition of zinc acetate and sodium carbonate. The sample was then centrifuged for 5 minute to precipitate the majority of unreacted GTP. The supernatant was loaded on a 2 ml alumina column and the synthesized cGMP was eluted with 10 ml of 50 mM Tris pH 7.5. The amount of radioactivity in the eluate was determined in a scintillation counter using Cherenkov emission, and used to quantify the amount of synthesized cGMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of dicyanocobinamide and protoporphyrin IX

Figure 2.
Caption NMR spectra of hybrid **Zn8** (mixture of regioisomers) in $\text{DMSO}-d_6$.

Figure 3.
Structure of hybrids **9** and **10**.

Figure 4.
Comparison of UV spectra of hybrid **8** superimposed with its components **2** and **6**.

Figure 5.

Activation of sGC by hybrids and its constituents. 0.5 μg sGC purified as described previously^[17] was incubated for 10 minutes with indicated concentrations of PpIX, dicyanocobyrinate hybrid or their constituents followed by determination of cGMP-forming activity as described in Experimental Section.^[7] The data are presented as means ±SD obtained from three independent experiments.

Scheme 1.
Synthesis of vitamin B₁₂ derivatives **2** and **3**.

Scheme 2.
 Synthesis of PpIX derivatives **6**, **7**, **Zn6** and **Zn7**.

Table 1

Optimization of CuAAC of **2** and **6** with **Zn6**.^[a]

Entry	M	Catalyst	Solvent	Time	Yield [%] ^[b]
1	2H	CuSO ₄ /Na ascorbate	BuOH/H ₂ O	12h	traces ^[c]
2	2H	CuI/TBTA ^[c]	BuOH/H ₂ O	12h	traces ^[c]
3	2H	CuI/TBTA ^[d]	BuOH/H ₂ O	12h	traces ^[c]
4	Zn	CuSO ₄ /Na ascorbate	BuOH/H ₂ O	4 days	10 ^[f,g]
5	Zn	CuI/TBTA ^[c]	BuOH/H ₂ O	12h	traces ^[f]
6	Zn	CuI/TBTA ^[c]	THF	12h	traces ^[f,g]
7	Zn	CuI/TBTA ^[c]	DMF	12h	traces ^[f,g]
8	Zn	(SiMes) ₃ CuBr	MeCN	12h	— ^[h]
9	Zn	(SiMes) ₃ CuBr	DCM	12h	— ^[h]
10	Zn	[(Phen)Cu(Ph ₃ P)]NO ₃	toluene	12h	— ^[h]
11	Zn	CuOAc	DCM	45min	75
12	2H	CuOAc	DCM	1h	n.d. ^[e]
13	Zn	CuOAc	toluene	12h	— ^[g]
14	Zn	CuOAc	THF	12h	traces ^[f,g]
15	Zn	CuOAc	CHCl ₃	12h	13 ^[f,g]
16 ^[i]	Zn	CuOAc	DCM	12h	70
17	Zn	CuOAc	DCM ^[j]	1.5h	68

Entry	M	Catalyst	Solvent	Time	Yield [%] ^[b]
18	Zn	CuOAc	DCM ^[k]	20min	40 ^[g]

^[a] Test reactions were performed on 0.02 mmol scale using 30 mol % of respective catalyst in 3ml of solvent at room temperature,

^[b] Isolated yield,

^[c] 30 mol % of TBTA was used,

^[d] 90 mol % of TBTA was used,

^[e] a mixture of **8** and **Cu8** was obtained,

^[f] conversion not full,

^[g] unidentified polar by-products occurred,

^[h] no conversion observed,

^[i] reaction performed at 0°C,

^[j] 9 ml of solvent was used,

^[k] 1ml of solvent was used.