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Brief Article

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Vitamin B_{12} Derivatives as Activators of Soluble Guanylyl Cyclase

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Supporting Information

ABSTRACT: Various newly prepared and previously known vitamin B_{12} derivatives have been studied as potential soluble guanylyl cyclase (sGC) activators. All compounds tested were found to activate the sGC enzyme, although to differing extents. The best results were obtained with the derivatives synthesized from *c*-lactone and possessing aliphatic amides in the *c*- and *d*-positions.

INTRODUCTION

Nitric oxide signaling is one of the fundamental pathways in mammalian physiology. It plays an important role in cardiovascular homeostasis, platelet function, angiogenesis, and neurotransmission. One of the key mediators of NO signaling is soluble guanylyl cyclase (sGC), a heme protein with high affinity for NO. The binding of NO to the heme moiety present in sGC enhances by 2 or 3 orders of magnitude its capacity to convert guanosine triphosphate (GTP) into a second messenger, cyclic guanosine monophosphate (*c*GMP). The resulting increase in intracellular cGMP concentration further triggers cGMP-dependent cellular effects that ultimately produce the characteristic physiological responses associated with NO signaling. For instance, sGC activation in smooth muscle cells leads to muscle relaxation and contributes to vasodilatation.[1,2] Likewise, NO-dependent activation of sGC inhibits adhesion of leukocyte and platelets to vascular walls and aggregation of platelets; it also decreases proliferation and migration of smooth muscles cells.[3] In this respect, activation and maintenance of sGC function is beneficial for maintaining normal vascular plasticity, preventing atherosclerosis,[4] thrombosis,[5] and stroke.[6] It also helps promote lung development in premature infants.[7] On the other hand, aberrations in NO-dependent pathways result in decreased vasodilatory function and contribute significantly to the development of cardiovascular, renal, pulmonary, and other pathologies associated with the diminished function of the endothelium.[8]

Not surprisingly, sGC has long been a therapeutic target and its function is currently regulated pharmacologically by various NO releasing organic nitrites, such as glyceryl trinitrate, isosorbide dinitrate, isosorbide 5-mononitrate. These agents are routinely used to manage angina pectoris, myocardial infarction, congestive heart failure, and hypertension.[9] Direct inhalation of NO gas is also used to manage persistent pulmonary hypertension in newborns.[10] Although the NO-based activation of sGC currently represents an accepted approach to producing useful clinical outcomes, a number of problems are associated with this generalized strategy. For instance, the effectiveness of inhaled NO is patient specific; some patients do not respond to the treatment, while a few develop adverse reactions.[11] The use of nitrovasodilators is also problematic, since patients often develop tolerance to nitrovasodilators.[12] Moreover, under inflammatory conditions, NO interacts with reactive oxygen species (ROS), leading to the formation of peroxynitrite ($ONOO^-$), which damages DNA, proteins, and lipids.[13] In these instances, NO-based targeting of sGC may produce more harm than benefit.

To overcome these problems, a number of compounds that activate sGC through NO-independent mechanisms have been described in the past decade.[14,15] As a general rule, these reported sGC regulators are thought to target the heme-containing regulatory domain of sGC. However, Martin and co-workers have recently reported that sGC could be activated by dicyanocobinamide **1** (Figure 1) via the catalytic domain.[16,17] They found that this vitamin B_{12} derivative not only activates the enzyme in vitro but also up-regulates its function in intact cells and isolated aortic rings. Subsequently, it was reported that dicyanocobinamide derivatives, with seven peripheral hydrophilic or hydrophobic substituents, are also effective as sGC activating agents.[18] Other derivatives, such as those containing a different number of amide substituents, were not included in these prior reports.

The present study has thus been carried out with a view to generalizing these previous results and obtaining basic structure−activity information. Toward this end, we have prepared several new cobalamin derivatives bearing various substituents at the periphery of the macrocycle and tested their

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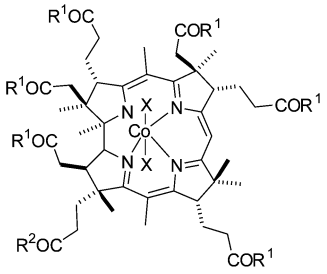


$R^1 = NH_2$	$R^2 = CH_2CH(OH)CH_3$	X = CN	1
$R^1 = OH$	$R^2 = OH$	X = CN,H_2O	2
$R^1 = OMe$	$R^2 = OMe$	X = CN	3
$R^1 = OMe$	$R^2 = OMe$	X = CN, H_2O	4
$R^1 = OMe$	$R^2 = OMe$		5

Figure 1. Hydrophilic and hydrophobic vitamin B_{12} derivatives.

sGC activation ability and those of related known compounds in vitro. As detailed below, some of the derivatives examined in this study displayed an sGC activating potential that was 240% greater than that of **1**.

To explore the effect that changes in structure would have on sGC activation, various acid, ester, and monamide derivatives of dicyanocobinamide, either known or newly prepared for this study, were selected for testing. In certain instances one or both of the cyano ligands present in **1** were replaced by water or removed altogether by reduction to the corresponding Co(II) species. For the sake of organization, the compounds are discussed according to the type of derivative prepared and the nature of the synthetic methodology employed to obtain the specific compound in question. Because the goal of the study was to obtain general insights, not all members of a given structural class were subjected to biological testing.

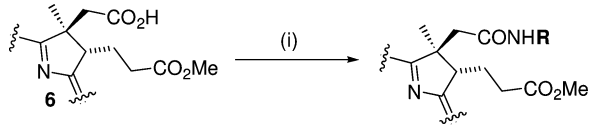
RESULTS AND DISCUSSION

Chemistry. As a first step in our structure−function analysis, the hydrophilic and hydrophobic cobalamin derivatives **2**−**5** were prepared and studied. This series was designed to give insights into the effect, if any, on activity that would come from increasing or decreasing the water solubility of the basic macrocyclic structure. To obtain a more water-soluble derivative, vitamin B_{12} was transformed into the corresponding heptaacid **2**. This was done following a literature procedure.[19]

In contrast, the presence of $-CO_2Me$ groups in these positions was expected to increase hydrophobicity, thus allowing increased penetration into a lipid membrane. To test this proposition, vitamin B_{12} was subject to hydrolysis followed by esterification with methanol. This produced the hydrophobic ester **3**,[20] a species that was subsequently transformed into the monoaqua complex **4** in accord with a literature procedure.[21] Compound **3** was also reduced to the corresponding Co(II) complex **5**. Having this latter complex in hand would provide insights into the role the redox state of the metal might play in regulating sGC activity.[21]

A second set of analogues was also studied. This series of compounds consisted of esterified vitamin B_{12} derivatives that retain the amide functionality present in **1** while varying the amine that makes up the amide. We sought to test the possibility that changing the amide moiety on a cobalamin would affect the activity of the resulting vitamin B_{12} derivative. Thus, a set of mono- and diamides possessing various terminal groups, including aliphatic, hydroxyl, methoxy, azide, and propargyl, as well as more complex amides, were prepared and examined as potential sGC acitivators. The compounds in question are shown in Schemes 1 and 2. They were chosen as targets since they embody the favorable structural features of amide **1** and hexaesters **3**−**5**. Their synthesis is detailed below.

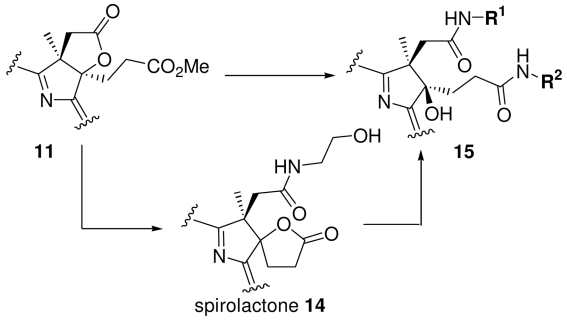
Scheme 1. Coupling of *c*-Acid 6 with Amines[a]



R= $-CH_2CO_2Me$	**7**
R= $-CH(CO_2Me)CH_2OH$	**8**
R= $-CH_2C_5H_4N$	**9**
R= -benzyl 18-crown-6 ether	**10**

[a](i) DEPC, R-NH_2, Et_3N, DMF.

Scheme 2. Synthesis of Mono and Diamides from *c*-Lactone



Derivative	R^1	R^2
12a	$-CH_2CH_2CH_2CH_2CH_2OH$	OMe
12b	$-CH_2CH_2CH_2OCH_3$	OMe
12c[27]	$-CH_2CH_2CH_2CH_3$	OMe
12d[27]	$-CH_2(CH_2)_6CH_3$	OMe
12e[27]	$-CH_2(CH_3)_2$	OMe
12f	$-CH_2(CH_2)_4CH_2N_3$	OMe
12g[27]	$-CH_2C{\equiv}CH$	OMe
12h	$-(CH_2CH_2O)_3CH_2CH_2N_3$	OMe
13a	$-C_2H_4OC_2H_4OH$	$-C_2H_4OC_2H_4OH$
13b[27]	$-CH_2CH_2CH_2OCH_3$	$-CH_2CH_2CH_2OCH_3$
13c	$-CH_2CH_2CH_2CH_3$	$-CH_2CH_2CH_2CH_3$
13d[27]	$-CH(CH_3)_2$	$-CH(CH_3)_2$
15[27]	$-CH_2CH_2OH$	$-C_2H_4OC_2H_4OH$

The initial set of monoamide derivatives was obtained starting from the *c*-acid **6**. Compound **6** was prepared from vitamin B_{12} via the lactone route.[22] It was transformed into its monoaquo species by washing with $HClO_4$[23,24] followed by coupling with various amines, using DEPC as a coupling reagent. This afforded amides **7**−**10** in good yields (Scheme 1).[25,26]

The amide bond could also be introduced via the direct ring-opening of the *c*-lactone **11** with amines (Scheme 2).[27] Since many of the species produced in this way cannot be accessed via the direct coupling method of Scheme 1, this ring-opening approach was considered to be a useful complement to more classic amide-forming strategies. Compound **15** was synthesized using a previously published method that incorporates the use of spirolactone **14**.

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While the large number of derivatives prepared in this way precluded the biological analysis of every compound (cf. Supporting Information for a full list of compounds and their characterization data), it did allow selected systems, representative of general compound type, to be chosen for study. The results of these studies are detailed later in this report.

All new compounds prepared in the context of this study were characterized by ^{1}H NMR spectroscopy, elemental analysis, and ESI MS (see Supporting Information).

Biological Results. Once the corrinoids described above were in hand, their effect on sGC activity was tested. Toward this end, purified enzyme was first incubated with different amounts of the cobinamide of interest. Then the cGMP-forming activity of sGC was determined using the assay described in the Experimental Section. While some cobinamides were ineffective, most of the tested compounds displayed some degree of dose dependent sGC activation. Representative dose−response curves are shown in Figure 2. Following this assay-based approach, the half maximal effective concentration (EC_{50}) for each of the activating cobinamides was determined (Table 1). Consistent with our previous report, dicyanocobinamide was found to activate sGC in a dose-dependent manner with a maximal 8-fold activation and an estimated EC_{50} = 34 μM being observed. These same experiments also revealed that changes in the cobinamide structure had a profound effect on the extent of sGC activaton. Some derivatives (e.g., **2**, **7**, **9**, and **10** in Table 1) displayed sGC activation values, which were lower than the standard against which they were benchmarked, namely, **1** dicyanocobinamide. A few others induced activation at a level similar to that of **1** (e.g., **3**, **5**, and **12g** in Table 1). However, several of these latter agents were characterized by an improved effective concentration. This makes them of interest.

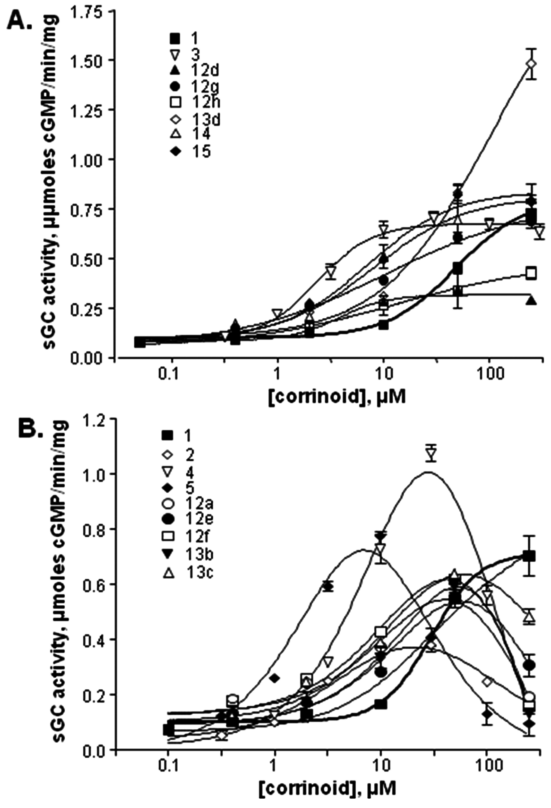

Figure 2. Stimulation of sGC activity by cobinamide derivatives. Purified human sGC (0.5 μg) was preincubated for 5 min with the indicated concentration of the cobinamide derivative in question and then tested for cGMP-forming activity using the [^{32}P]GTP assay as described in the Experimental Section. Data are shown as the mean ± SD from two independent experiments performed in triplicate. Dose−response curves display features consistent with a monophasic (A) or a biphasic (B) regulation of sGC activity. The symbols are the mean ± SD (n = 6), while the solid lines are nonlinear regression curve fits obtained using the sigmoidal dose−response (A) or two-site competition (B) algrithms of the GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, U.S.). See text for discussion.

Table 1. Vitamin B_{12} Derivatives with sGC-Stimulating Properties

entry	derivative	EC_{50} (μM)[a]	IC_{50} (μM)[a]	activity ratio relative to **1**
1	**1**	34.2	na	1
2	**2**	20.5	547	0.48
3	**3**	9.3	na	0.92
4	**4**	14.1	39.8	1.34
5	**5**	4.5	12.6	0.96
6	**7**	17.4	na	0.71
7	**8**	20.5	547.6	0.74
8	**9**	5.3	na	0.53
9	**10**	10.1	na	0.42
10	**12a**	25.1	158.5	0.77
11	**12b**	12.6	316	0.84
12	**12c**	16.4	204.9	0.91
13	**12d**	3.8	na	0.57
14	**12e**	25	251	0.8
15	**12f**	34.7	316.2	0.88
16	**12g**	11.9	na	1.13
17	**12h**	12.7	na	0.71
18	**13a**	5.6	na	0.28
19	**13d**	101	na	2.5
20	**14**	9	na	1.31
21	**15**	7.5	na	1.37

[a] EC_{50} and IC_{50} were calculated via nonlinear regression using GraphPad Prism 5. na: not applicable.

Finally, as shown in Figure 2 and Table 1, some cobinamide derivatives (**13d**, **14**, and **15**) produced a higher level of activation than **1**. On the basis of the combined total of the data, we conclude that cobalamin diamide derivatives, particularly those bearing branched and long-chain alky substituents, are promising candidates for sGC activation.

A more detailed analysis of the sGC-activating cobinamides revealed that the derivatives of this study may be divided into two groups. One set displays a typical one phase dose-dependent activation of sGC (Figure 2A). As shown in Table 1, many of these compounds had an improved affinity toward sGC, as demonstrated by decreased EC_{50} (Table 1). Another group of compounds displayed a bell-shaped dose-dependent activation curve (Figure 2B). Such biphasic behavior is consistent with the existence of a secondary cobinamide binding site with lower affinity. To the extent this supposition is correct, this second site exerts an inhibitory effect on sGC. The EC_{50} for the activating site and the IC_{50} for the putative inhibitory site are shown in Table 1. In general, the diamides displayed enhanced inhibitory activity compared to the analogous monoamides.

Previous studies served to demonstrate that dicyanocobinamide activates sGC through a mechanism different from those of other sGC activators.[16] Unlike nitric oxide or any of the NO-independent activators previously described, dicyanocobina-

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mide activates sGC by directly targeting the catalytic domain of the enzyme.[16]

The catalytic domain of sGC is composed of the C-terminal regions of the α and β subunits, both of which are required for catalysis. These subunits have a 60% homology in the catalytic region, which leads to the prediction that the catalytic domain has a pseudosymmetric structure.[28] Such a presumed pseudosymmetric structure may account for the existence of two cobinamide-binding sites with different affinities. The existence of a two-site arrangement is reminiscent of the substrate and pseudosubstrate organization predicted for GTP analogues.[29] Nevertheless, further study will be required before the exact localization of the cobinamide-binding sites can be determined.

CONCLUSIONS

Existing NO-independent activators were shown to be effective in a variety of experimental animal models and to have therapeutic promise for a range of cardiovascular and noncardiovascular disorders. These latter disorders include arterial and pulmonary hypertension, peripheral arterial disease, heart failure, renal fibrosis, erectile dysfunction, peripheral arterial occlusive disease, atherosclerosis, restenosis, and thrombosis.[14,15] Previous investigations served to demonstrate that dicyanocobinamide synergistically enhances the effects of all classes of NO-independent sGC regulators, leading us to suggest that corrin species may be used in combination with these previously known agents to enhance their therapeutic potential. However, to be effective, relatively high concentrations of dicyanocobinamide are required. This is likely to limit its pharmacological use. The compounds presented in this report display improved effective concentrations and/or enhanced sGC activating potential. Future investigations will test if this augmented effectiveness in vitro translates into improved sGC activating potency in vivo.

EXPERIMENTAL SECTION

Chemistry. General and Materials. All solvents and chemicals used in the syntheses were of reagent grade and were used without further purification. Full description of ^{1}H and ^{13}C NMR data for all new compounds are in the Supporting Information. Tested compounds had >95% chemical purity as measured by HPLC analysis. Vitamin B_{12} was purchased from Aldrich.

Preparation of 7. *c*-Acid (**6**)[22] (15 mg, 0.014 mmol) was dissolved in dimethylformamide (DMF, 1 mL) and cooled to 0 °C using an ice bath under a nitrogen atmosphere. DEPC (9 μL, 0.06 mmol) was added to the solution followed by Gly-OMe (4.0 mg, 0.04 mmol) and triethylamine (8 μL, 0.12 mmol). The mixture was stirred for 6 h at 0 °C and then 17 h at room temperature under a nitrogen atmosphere. The mixture was then diluted with dichloromethane (DCM) and washed with water. The organic layer was separated, dried over anhydrous Na_2SO_4, and evaporated to dryness. The product was purified using DCVC, 2.5% EtOH in DCM. After recrystallization from hexanes/AcOEt, **7** was isolated as a purple solid (14 mg, 86%). R_f = 0.50, 5% EtOH in dichloromethane (DCM). LRMS ESI (m/z) calcd for $C_{55}H_{76}CoN_6O_{15}$ $[M - CN]^+$ 1120.1; found 1120.1. UV/vis CH_2Cl_2, λ_{max} nm (ε, L·mol^{-1}·cm^{-1}): 588 (1.05×10^4), 5.49 (8.62×10^3), 422 (2.72×10^3), 371 (2.78×10^4), 317 (9.52×10^3), 279 (1.09×10^4).

Preparation of 8. Following the procedure for **7**, **8** was isolated as a purple solid (51 mg, 54%). R_f = 0.50, 5% EtOH in DCM. LRMS ESI (m/z) calcd for $C_{56}H_{78}CoN_6O_{16}$ $[M - CN]^+$ 1149.5; found 1149.9. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 589 (9.791×10^3), 550 (7.64×10^3), 424 (2.55×10^3), 371 (2.65×10^4), 317 (8.99×10^3), 279 (1.00×10^4).

Preparation of 9. Following the procedure for **7**, **9** was isolated as a purple solid (190 mg, 45%.). MALDI-TOF-MS (m/z): $[M - 2CN]^+$, 1112. UV/vis CH_2Cl_2, nm: 278, 312, 370, 421, 510(sh), 547, and 587.

Preparation of 10. Following the procedure for **7**, **10** was isolated as a purple solid (74 mg, 48%). MALDI-TOF-MS (m/z): $[M - CN]^+$, 1344. UV/vis CH_2Cl_2, nm: 371, 421, 510 (sh), 547, and 587.

Preparation of 12a. Following a reported procedure,[27] **12a** was isolated as a purple solid (14 mg, 87%). R_f = 0.37, 5% EtOH in DCM. HRMS ESI (m/z) calcd for $C_{57}H_{82}CoN_6O_{15}$ $[M - CN]^+$ 1149.5164; found 1149.5177. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 281 (9.50×10^3), 319 (9.45×10^3), 372 (2.68×10^4), 426 (2.68×10^3), 553 (8.29×10^3), 591 (1.01×10^4).

Preparation of 12b. Following a reported procedure,[27] **12b** was isolated as a purple solid (12 mg, 75%). R_f = 0.36, 5% EtOH in DCM. HRMS ESI (m/z) calcd for $C_{56}H_{80}CoN_6O_{15}$ $[M - CN]^+$ 1135.5028; found 1135.5008. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 281 (9.73×10^3), 319 (9.57×10^3), 372 (2.77×10^4), 426 (2.93×10^3), 554 (8.50×10^3), 593 (1.06×10^4).

Preparation of 13b. Following a reported procedure,[27] **13b** was isolated as a purple solid (9 mg, 52%). R_f = 0.13, 5% EtOH in DCM. HRMS ESI (m/z) calcd for $C_{59}H_{87}CoN_7O_{15}$ $[M - CN]^+$ 1192.5572; found 1192.5586. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 281 (9.61×10^3), 319 (9.46×10^3), 372 (2.65×10^4), 425 (3.15×10^3), 553 (8.40×10^3), 591 (9.82×10^3).

Preparation of 12f. Following a reported procedure,[27] **12f** was isolated as a purple solid (22 mg, 72%). R_f = 0.32, 5% EtOH in DCM. HRMS ESI (m/z) calcd for $C_{54}H_{76}CoN_6O_{15}$ $[M - CN]^+$ 1188.5389; found 1188.5386. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 281 (8.85×10^3), 319 (9.11×10^3), 372 (2.26×10^4), 427.5 (3.12×10^3), 556 (7.15×10^3), 592 (8.62×10^3).

Preparation of 12h. Following a reported procedure,[27] **12h** was isolated as a purple solid (33 mg, 45%). R_f = 0.48, 5% EtOH in DCM. HRMS ESI (m/z) calcd for $C_{60}H_{87}CoN_9O_{17}$ $[M - CN]^+$ 1264.5512; found 1264.5546. UV/vis CH_2Cl_2, λ_{max}, nm (ε, L·mol^{-1}·cm^{-1}): 281 (9.42×10^3), 319 (9.09×10^3), 372 (2.57×10^4), 426 (2.92×10^3), 554 (7.84×10^3), 593 (9.96×10^3).

Biology. Purification of Recombinant Human sGC Enzyme and Determination of Its Activity. Recombinant human sGC enzyme was purified as described previously from Sf9 cells infected with baculoviruses expressing α1 and β1 sGC subunits.[30] Only preparations with NO-induced activity of >5 μmol min^{-1} mg^{-1} were used for the present studies. To evaluate the effect of synthesized cobinamides on sGC, 0.5 μg of sGC was preincubated for 5 min at 25 °C with the indicated concentration of the compound. This was followed by testing in the $\alpha[^{32}P]$GTP → $[^{32}P]$cGMP conversion assay as described previously.[17]

ASSOCIATED CONTENT

Supporting Information

Experimental procedures and characterization data and ^{1}H and ^{13}C NMR spectra for all new compounds; experimetnal procedure for enzyme purification and assay of sGC acitivity in vitro. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

sGC, soluble guanylyl cyclase; DIC, diisopropylcarbodiimide; DEPC, diethyl cyanophosphate

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