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Metallophyte wastes and polymetallic catalysis: a promising combination in green chemistry. The illustrative synthesis of 5'-capped RNA†

Yann Thillier,^a Guillaume Losfeld,^b Vincent Escande,^b Christelle Dupouy,^a Jean-Jacques Vasseur,^a Françoise Debart^{*a} and Claude Grison^{*b}

The compatibility of metallic catalysts derived from plants was studied for the chemical solid-phase synthesis of a high added-value biomolecule: 5'-capped RNA (GpppRNA or ^{7m}GpppRNA) involved in many essential biological processes. These molecules are of great interest for biologists for structural and mechanistic studies of their complexes with RNA capping enzymes. Several polymetallic catalysts were prepared from various metallophytes species growing in mine sites in a context of phytoremediation programs. Their catalytic efficacy was evaluated for the coupling reaction of guanosine diphosphate (GDP) with a preactivated 5'-monophosphate hexathymidylate (pT₆) or a pRNA to form 5'-GpppT₆ or 5'-GpppRNA respectively. The direct coupling of ^{7m}GDP with pRNA was also investigated. With Zn-enriched catalysts obtained from Zn metallophytes, the capping yields were better than those obtained with conventional catalyst ZnCl₂. Interestingly with Ni-enriched plant extracts the yield was higher than with NiCl₂, a polymetallic catalysis was certainly involved. With this plant-inspired catalysis, metallophytes wastes become efficient and greener polymetallic catalysts. Finally, environmental benefits and effectiveness can be combined.

Introduction

The concept of green chemistry holds great promise for reducing the negative environmental impacts of organic synthesis. The use of chemical processes designed to reduce waste products, to facilitate efficient reuse and recycling, to optimize high atom-economy, and to use reagents that are less hazardous to human health and the environment have contributed to significant advances in the development of clean processing pathways. The search for starting materials derived from renewable resources and abundant bio-based materials are among the relevant strategies under development in chemistry today. However, green chemistry implies contributing to solving or mitigating environmental problems as well as improving industrial efficiency. It can play a key role in this area for example through the development of innovative solutions.

The European Environmental Agency estimates that 3 million sites in European Union countries are polluted. The

most common harmful contaminants are heavy metals. The discovery of metal hyperaccumulator plants able to extract and concentrate heavy metals from contaminated soils in their leaves and shoots provides an attractive opportunity to remove them from polluted soils and ecosystems. The use of such plants has led to the development of various phytoextraction techniques.^{1–5} Today, using vascular plants to 'clean up' the environment may potentially be used in large-scale cost-effective projects, and have a high degree of public approval.^{6–9} Benefits for the environment and for human health are well quantified. This has resulted in a clear European-wide call for greater efforts to be made in ecological remediation and restoration of polluted sites. However, the financial aspect of phytoextraction remains a difficulty, because existing techniques of phytotechnology lack real economic outlets, and thus the costs outweigh the economic benefits. In other words, there is no clear business case for industrial stakeholders and public funds are not currently made available for this purpose.

Our previous research has demonstrated that phytoextraction of heavy metals can constitute the starting point of an original and attractive approach for 'green' or 'environmentally-friendly' heterogeneous catalysis.^{10–12} Notably, the synthetic potential of these new systems was illustrated through model reactions of industrial interest, Friedel–Crafts and Lucas reactions. In the present contribution, we demonstrate that these novel, 'bio-inspired' metallic catalysts promote the

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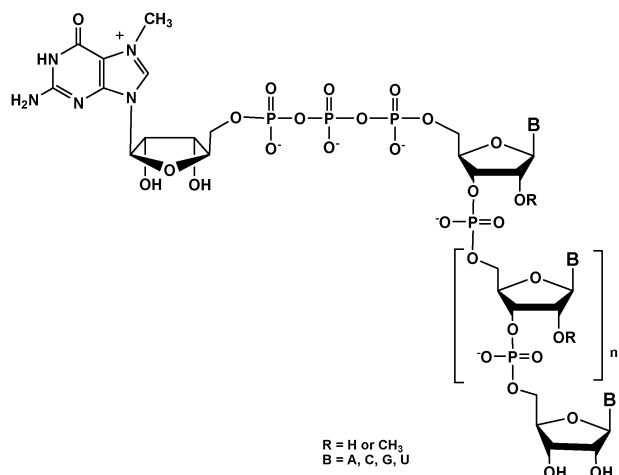


Fig. 1 mRNA cap structure ^{7m}Gppp.

synthesis of high added-value molecules characterized by structural complexity. A key example is the chemical functionalization of RNA with cap structures at their 5'-end (Fig. 1). The cap moiety consists of a *N*⁷-methylguanosine nucleoside (^{7m}GpppN) linked to the 5'-terminal nucleoside of the pre-mRNA via a 5'-5' triphosphate bond.^{13,14} This modification is critical for efficient translation,¹⁵ for limiting RNA degradation by cellular 5' exonucleases and for avoiding recognition of mRNA as "nonself" by the innate immunity machinery.¹⁶ Convenient access and availability of large quantities of capped RNA (^{7m}GpppRNA) are of great interest for biologists for structural and mechanistic studies of their complexes with RNA capping enzymes. *In vitro* enzymatic methods cannot produce sufficient amounts of ^{7m}GpppRNA of defined sequences. Although a few chemical methods had been described, most of them produce a very limited amount of capped RNA.^{17,18} Therefore the need of an universal and efficient method to obtain these synthetic RNA in significant quantities and high purity was critical. To overcome this bottleneck, we have recently developed an efficient strategy for the production of capped RNA with high yields and without any limitation concerning the nucleotide present at the 5'-end of the RNA substrate.¹⁹ This consists in the combination of chemical solid-phase synthesis of GpppRNA followed by enzymatic *N*⁷-methylation. In this method, the functionalization at 5'-end of RNA with the cap structure involves the use of zinc chloride for the coupling reaction of guanosine diphosphate (GDP) with a preactivated 5'-monophosphate oligonucleotide to form GpppRNA. Here we report on the capping reaction using innovative and various metal chlorides, which are derived from different metallophyte species to functionalize RNA at their 5'-end with the cap structures Gppp or ^{7m}Gppp (Fig. 1). Furthermore for the first time we describe a straightforward chemical synthesis to obtain ^{7m}GpppRNA. The compatibility of bio-based catalysts was studied for the chemical synthesis of various lengths and sequences of natural and chemical modified DNA and RNA.

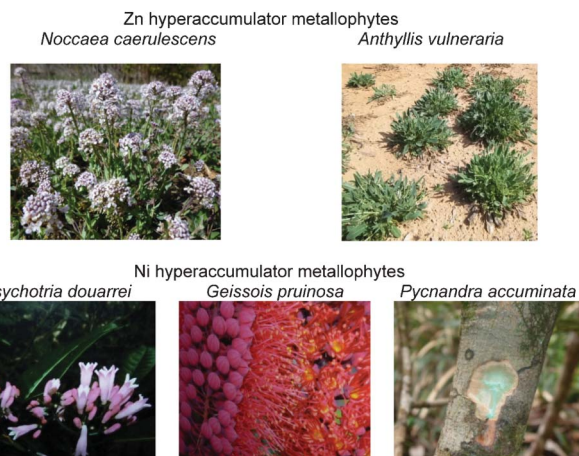


Fig. 2 Metallophytes species for polymetallic catalysis.

Results and discussion

Green polymetallic catalysts derived from various metallophytes species: preparation and analysis

The selection of hyperaccumulator plants was undertaken in the context of our ongoing phytoremediation programs (Fig. 2). Zn hyperaccumulating leaves were derived from *Noccaea caerulea* and *Anthyllis vulneraria*. They were collected from plants growing on the Les Avinières mine site, at Saint-Laurent-Le-Minier (Gard) in the Mediterranean climate region of southern France. Ni hyperaccumulating leaves were derived from *Psychotria douarrei*, *Geissois pruinosa* and *Pycnanthus accuminata*. They were collected from plants growing in the Southern Province of the subtropical Pacific island of New Caledonia.

Leaves were harvested before flowering, air-dried and crushed. The obtained solid was calcined at 400 °C for 5 h and the resulting powder was added to a 5 M HCl solution. After heating the mixture at 60 °C for 2 h, then filtration on celite, the resulting solutions, composed of different metal chlorides, were then concentrated under vacuum. Dry residues were either used crude or partially purified in order to decrease the concentration of alkali and alkaline earth metals in the catalytic solid. When Ni hyperaccumulating metallophytes (*P. douarrei* and *G. pruinosa*) were used, a chelating resin allowed Ni concentration and partial elimination of undesired metals ions. Thus, elution of alkali and alkaline earth metals was performed with HCl at pH 2.5 whereas transition metals were eluted with 12 M HCl. With Zn hyperaccumulating metallophytes (*N. caerulea* and *A. vulneraria*), IRA 400 ion exchange resin was considered and its use resulted in adsorption of Zn^{II} on the resin, and elution of alkali and alkaline earth cations.

Treatment with 0.5 M HCl eliminated a part of Fe^{III} fixed on the resin before the elution of heavy metals (Zn^{II}, Cd^{II}, Pb^{II}) with 0.005 M HCl. Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the composition of the various plant extracts obtained. Detailed results are presented in Table 1.

Table 1 Detailed composition of catalytic solids derived from metallophyte species

Entry	Catalysts	Concentration (ppm)									
		Mg	Al	Ca	Mn	Fe	Ni	Cu	Zn	Cd	Pb
1	<i>N. caerulescens</i> extract										
	-crude	34 769	10 294	330 362	594	11 448	584	409	89 157	7119	21 050
2	-purified (fraction 1)	49 513	209	358 327	190	1002	506	58	524	140	131
3	-purified (fraction 3)	30 130	8530	180 873	3598	30 740	3598	655	405 477	6400	36 078
	<i>N. caerulescens</i> + <i>A. vulneraria</i> extract										
4	-crude	28 202	14 878	134 925	936	27 275	2985	523	91 232	4845	29 249
	<i>P. douarrei</i> extract										
5	-crude	20 768	5419	96 731	1402	5570	80 153	299	7902	80	703
6	-purified	22 320	2250	91 212	1596	2808	184 600	401	6863	131	942
	<i>G. pruinosa</i> extract										
7	-crude	76 265	5302	119 756	1066	7882	73 863	551	12 524	215	2808
8	-purified	31 969	4764	87 667	3155	4589	150 395	406	9597	153	2083
	<i>P. accuminata</i> extract										
9	-crude	22 519	4002	40 659	1399	5791	115 163	115	2344	224	1393

According to Pearson's HSAB (hard and soft (Lewis) acids and bases) principle, the catalytic solids generated from metallophytes led to modulation of the hard/soft ratio. The obtained catalytic solids could be distinguished according to three types of Lewis acid level: the purified *N. caerulescens* extract, called fraction 1 (Table 1, entry 2), led to a Pearson's "Hard Lewis Acid" mixture, because the Mg^{2+} , Ca^{2+} , Al^{3+} , Fe^{3+} contribution represented 99.6% of the cationic mixture.

Purified *N. caerulescens* extract, called fraction 3 (Table 1, entry 3), purified *P. douarrei* (Table 1, entry 6) and *G. pruinosa* (Table 1, entry 8) extracts led to a Pearson's "Borderline Lewis Acid" composition, with a mixture of borderline and soft Lewis acids ($\text{Mn}^{2+} + \text{Ni}^{2+} + \text{Cu}^{2+} + \text{Zn}^{2+} + \text{Cd}^{2+} + \text{Pb}^{2+} = 62, 64, 66\%$). Crude extracts were constituted by miscellaneous cations composition with respect to Pearson's classification system.

Chemical synthesis of hexathymidylate with 5'-cap structure (GpppT₆) (Table 2, entries 1-12)

Before the capping reaction on RNA sequences of biological interest, we tested various catalytic metals derived from metallophyte species with a DNA homosequence as a model. Therefore we initiated the study with the synthesis of 5'-guanosyl triphosphate hexa-2'-deoxythymidylate (GpppT₆) **5**, which was prepared at 1 μmol scale on solid support following the same route (Scheme 1) as recently described.¹⁹ The solid-supported T₆ **1** was assembled by the standard automated phosphoramidite method using a controlled pore glass support (CPG). After elongation, T₆ **1** was converted into its 5'-H-phosphonate derivative **2** which was activated as its phosphoroimidazolide **3** by amidative oxidation with quantitative yield.

The key step is the coupling between the commercial guanosine diphosphate (GDP) and hexathymidyl 5'-phosphoroimidazolide **3** linked to the solid support. The general mechanism is based on a nucleophilic attack of the GDP phosphoryl moiety on the 5'-phosphoramidate **3** displacing the imidazolide group. The ideal conditions to obtain the triphosphate bond were satisfied if:

- unprotected and poorly soluble GDP sodium salt in organic solvent was converted into GDP bis(tri-*n*-butylammonium) to

pass into solution by association with a divalent metal chloride;

- the metal salt activated phosphoramidate **3** to enhance the coupling reaction with GDP;

- the activation conditions should be compatible with fragility of the GDP and the formed triphosphate bridge.

Among several divalent metal salts, ZnCl_2 in anhydrous DMF has been found to be the most efficient. This result illustrated the interest of Pearson's "Borderline Lewis Acids" in this reaction. However, the reaction remains quite a delicate problem. This could be accomplished by careful and delicate reaction condition control and moderate yields. In the context of our investigations, we aimed to improve the reaction efficiency with our 'eco-friendly' and non-conventional catalysts. From a statistical point of view, it appeared reasonable to expect that a mixture of different metal halides could better interact with three distinct coordination sites than a single metal cation; consequently, a polymetallic catalyst could strongly promote the reaction sequence.

Substitution of the imidazole by GDP (0.14 mmol) was successfully accomplished at 30 °C for 18 h in dry DMF in the presence of a divalent metal salt (0.2 mmol) to give the capped GpppT₆ **4** still anchored to the solid support (1 μmol). After removal of the capping solution from the synthesis reactor using a mix of water and EDTA followed by several washes with CH_3CN , the capped GpppT₆ were deprotected and released from solid support. First, cyanoethyl groups were eliminated from the phosphates with 1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution in dry CH_3CN for 3 min. Then, treatment with 30% aqueous ammonia at room temperature cleaved GpppT₆ **5** from the CPG support. The conversion yields of T₆ into the desired capped GpppT₆ were calculated by integration of the major peaks corresponding to the capped T₆ in the ion-exchange HPLC chromatograms of the crude materials (Fig. 3).

The capping yield could reach up to 66% (Table 2, entry 6). Further characterization was completed by MALDI-TOF mass spectrometry (Table 2).

A global analysis shows the interest of these new systems to facilitate catalysis during the coupling reaction. All crude extracts allowed the complete dissolution of GDP and

Table 2 Data for synthesized 5'-GpppT₆, 5'-GpppRNAs and 5'-^{7m}GpppRNAs

Entry	Catalysts ^a	5'-sequence-3'	Yield (%) ^b	Crude yield ^c	Isolated yield ^d	Molecular formula	Calc. m/z ^e	Found m/z ^e
1	ZnCl ₂	GpppT ₆	55	498	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.29
2	NiCl ₂	GpppT ₆	26	421	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.61
<i>N. caerulescens</i> extract (Zn-enriched)								
3	crude	GpppT ₆	31	430	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.34
4	purified (f1)	GpppT ₆	0	351	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	—
5	purified (f3) ^f	GpppT ₆	55	487	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.25
6	purified (f3)	GpppT ₆	66	512	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.65
<i>N. caerulescens</i> + <i>A. vulneraria</i> extract								
7	crude	GpppT ₆	6	402	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.57
<i>P. douarrei</i> extract (Ni-enriched)								
8	crude	GpppT ₆	26	437	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.36
9	purified ^f	GpppT ₆	56	378	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.80
<i>G. pruinosa</i> extract (Ni-enriched)								
10	crude	GpppT ₆	18	423	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.34
11	purified	GpppT ₆	42	461	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.29
<i>P. accuminata</i> extract (Ni-enriched)								
12	crude	GpppT ₆	33	456	nd	C ₇₀ H ₉₃ N ₁₇ O ₅₇ P ₈	2267.35	2267.19
13	ZnCl ₂	GpppAGUUGUUAGUCUUACUGG	46	331	73	C ₁₅₁ H ₂₂₅ N ₇₀ O ₁₄₁ P ₂₀	6254.57	6254.86
14	ZnCl ₂	GpppAUUAUUA	45	408	138	C ₆₇ H ₈₃ N ₂₆ O ₅₃ P ₈	2348.31	2348.32
15	ZnCl ₂	^{7m} GpppAUUAUUA	38	303	89	C ₆₈ H ₈₅ N ₂₆ O ₅₃ P ₈	2362.34	2362.67
<i>N. caerulescens</i> extract (Zn-enriched)								
16	purified (f3)	GpppA _{OMe} GUUGUUAGUCUUACUGGA	43	491	92	C ₁₉₂ H ₂₃₉ N ₇₅ O ₁₄₅ P ₂₁	6597.80	6597.45
17	purified (f3)	GpppAUUAUUA	32	372	102	C ₆₇ H ₈₃ N ₂₆ O ₅₃ P ₈	2348.31	2348.15
18	purified (f3) ^g	^{7m} GpppAUUAUUA	33	312	54	C ₆₈ H ₈₅ N ₂₆ O ₅₃ P ₈	2362.34	2362.59
<i>P. douarrei</i> extract (Ni-enriched)								
19	purified	GpppA _{OMe} GUUGUUAGUCUUACUGGA	34	422	81	C ₁₉₂ H ₂₃₉ N ₇₅ O ₁₄₅ P ₂₁	6597.80	6597.03
20	purified	GpppAUUAUUA	23	344	73	C ₆₇ H ₈₃ N ₂₆ O ₅₃ P ₈	2348.31	2348.32
21	purified	^{7m} GpppAUUAUUA	7	302	nd	C ₆₈ H ₈₅ N ₂₆ O ₅₃ P ₈	2362.34	2362.36

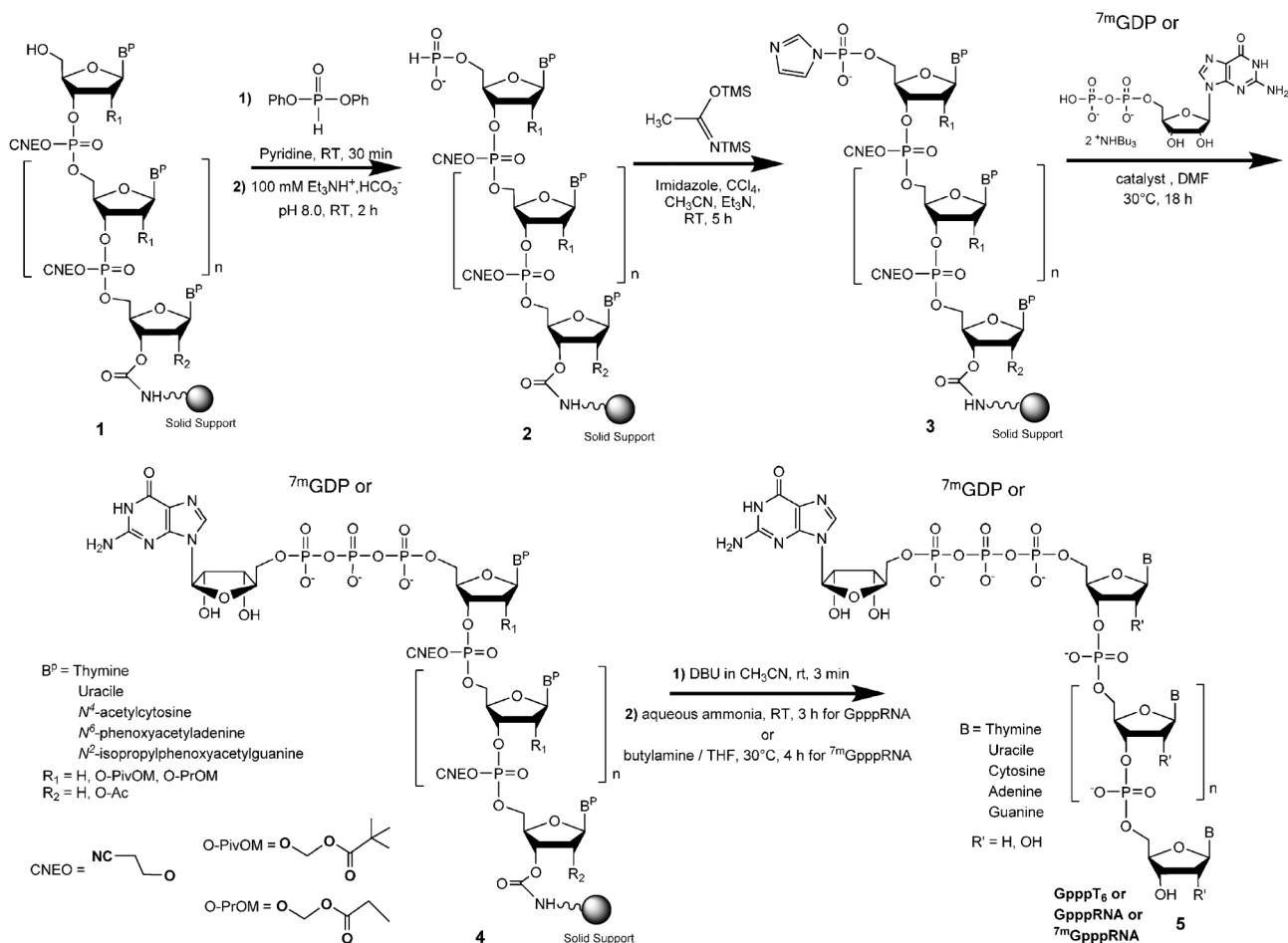
^a Final concentration in the reaction mixture of [ZnCl₂] = [NiCl₂] = 0.4 M in DMF. ^b Percentage yield of oligonucleotide in the crude as calculated from the integration of the IEX chromatogram. ^c nmol crude total material. ^d nmol pure product GpppRNA obtained after HPLC purification. nd: not determined. Oligonucleotides (entries 1–12, 20) were not HPLC purified. ^e MALDI-TOF characterization in negative mode. ^f The catalyst-GDP solution was not centrifuged, but the final mixture was eluted on Sephadex. ^g The acidity of the catalyst was neutralized by refluxing the catalytic extract in dioxane to prevent depurination.

promoted the substitution of imidazolide (Table 2, entries 3, 8, 10 and 12 with 31%, 26%, 18% and 33% yield, respectively). Interesting results were obtained when the clear solution resulting from centrifugation of catalytic extracts and GDP in DMF had been applied (Table 2, entries 6 and 11 with 66% and 42% respectively). The ability of metallophyte extracts to perform catalysis depends critically on metal ion composition. Excluding the inactive fraction 1 derived from *N. Caerulescens* (Table 2, entry 4), all purified fractions (Table 2, entries 5, 6, 9, 11) led to comparable results to conventional catalysts ZnCl₂ and NiCl₂ (Table 2, entries 1 and 2). It is noteworthy that the purified fraction 3 derived from *N. caerulescens* (Table 2, entry 6) was better than commercial ZnCl₂ (66% and 55%, respectively) (Fig. 3, Table 2, entries 1 and 3). Catalysts derived from Ni hyperaccumulating plants (Table 2, entries 8–12) should also be noted since *G. pruinosa* and *P. douarrei* led to expected products with satisfactory yields ranging from 42% (Table 2, entry 11) to 56% (Table 2, entry 9) whereas it is well known that NiCl₂ is a poor acid-catalyst. Besides to date, no such capping reaction catalyzed by NiCl₂ has ever been described.

Crude *P. douarrei* extract (Table 2, entry 8) had a similar catalytic activity to NiCl₂ (Table 2, entry 2) with 26% coupling yield. Interestingly, despite of its weaker interest in our phytoremediation program for Ni-rich metallophytes, *P.*

accuminata crude extract gave the best coupling yield (33%). The *P. douarrei* and *G. pruinosa* purified extracts (Table 2, entries 9 and 11) were more active than commercial NiCl₂. With the purified fraction issued of *P. douarrei* extract (Table 2, entry 9), the coupling yield with GDP was increased twice as much as compared with NiCl₂ (56% instead of 26%) and reached the 55% yield obtained with ZnCl₂ (Table 2, Fig. 3). It may be concluded that a synergetic effect exists between the present Lewis acids which improves catalytic performance, when a mixture of borderline and soft Lewis acids was made (purified extracts). These observations agree with Mikkola and *et al.*, who found that a combination of two metal halides leads to more active systems than individual components in the 5'-cap nucleotides synthesis.²⁰

As discussed above, their impact on catalysis is threefold. Thus, understanding the mechanisms underlying polymetallic catalysis is difficult. In order to learn more about these results, we have compared the ratio of expected and observed side-products for each of the catalytic compositions. Obtained results suggest that Ni metallophytes favoured GppT₆ formation instead of the expected GpppT₆ (Table 3). So, it seems that Ni catalysts promote partial hydrolysis of GDP into GMP before coupling reaction. However the association of Ni and other metals, more efficient than sole NiCl₂, can be explained by the binding of two metal ions to two adjacent Lewis base centers



Scheme 1 Solid-phase synthesis of 5'-GpppT₆, 5'-GpppRNA or 5'- 7^m GpppRNA.

of phosphoroimidazolidate **3** (Fig. 4). In view of the excellent affinity of the imidazole ring for Ni^{2+} , we can suppose an interaction between the heterocycle and NiCl_2 , which strengthens the leaving group capacity of imidazole. This effect is added to the coordination between other metals and the phosphate group that increases electrophilicity of the activated phosphoryl ligand of **3**. This double effect promotes the coupling reaction.

N. caerulea + *A. vulneraria* crude extract (Table 2, entry 7) led to poor yield (6%). This result can be correlated with the amount of AlCl_3 (Table 1, entry 4). Finally, *N. caerulea* purified extract (Table 2, fraction 1, entry 4) didn't promote the coupling reaction, since it is known that Mg^{2+} and Ca^{2+} have high affinity for the phosphate oxygen (Table 1, entry 2). Hard Lewis Acid mixture cannot activate the P-N bond. It can be concluded that essential cations for plant growth (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cu^{2+}) are not useful in the aimed reaction, whereas Zn^{2+} , Ni^{2+} , Cd^{2+} and Pb^{2+} which result of heavy-metal hyperaccumulation promote the coupling reaction. Overall, these results show that the metallophyte species led to the preparation of an interesting polymetallic composition, which allowed coordination not only with the phosphate oxygen but also with the cyclic imidazole of the substrate.

Chemical synthesis of RNA with 5'-cap structure (GpppRNA or 7^m GpppRNA) (Table 2, entries 13–21)

Encouraged by the efficiency of these catalysts, the reaction was extended to the synthesis of Gppp6-mers (Table 2, entries 14, 17, 20), Gppp19-mers (Table 2, entries 13, 16, 19) and 7^m Gppp6-mers (Table 2, entries 15, 18, 21) RNA heteropolymers with the two best catalytic systems: *N. caerulea* and *P. douarrei* extracts.

Regarding the synthesis of 5'-capped RNA on solid support, the instability of N^7 -methylguanosine under acidic and basic conditions and RNA sequence fragility are additional difficulties of the second part of this work. Indeed, because of the positive charge on the N^7 -methylguanosine (Fig. 1), the nucleoside is hydrolytically less stable than standard purine nucleosides. Under basic conditions used for standard RNA deprotection, the opening of the imidazole ring of the 7-methylguanine would occur. For this reason, the synthesis of 7^m GpppRNA completely achieved on solid support excludes the ammonia treatment to deprotect and to release 7^m GpppRNA. Therefore the strategy for RNA assembly to provide 7^m GpppRNA was different from the pivaloyloxymethyl (PivOM) technology used for RNA synthesis to get GpppRNA.²¹ The major feature of this technology developed by our group

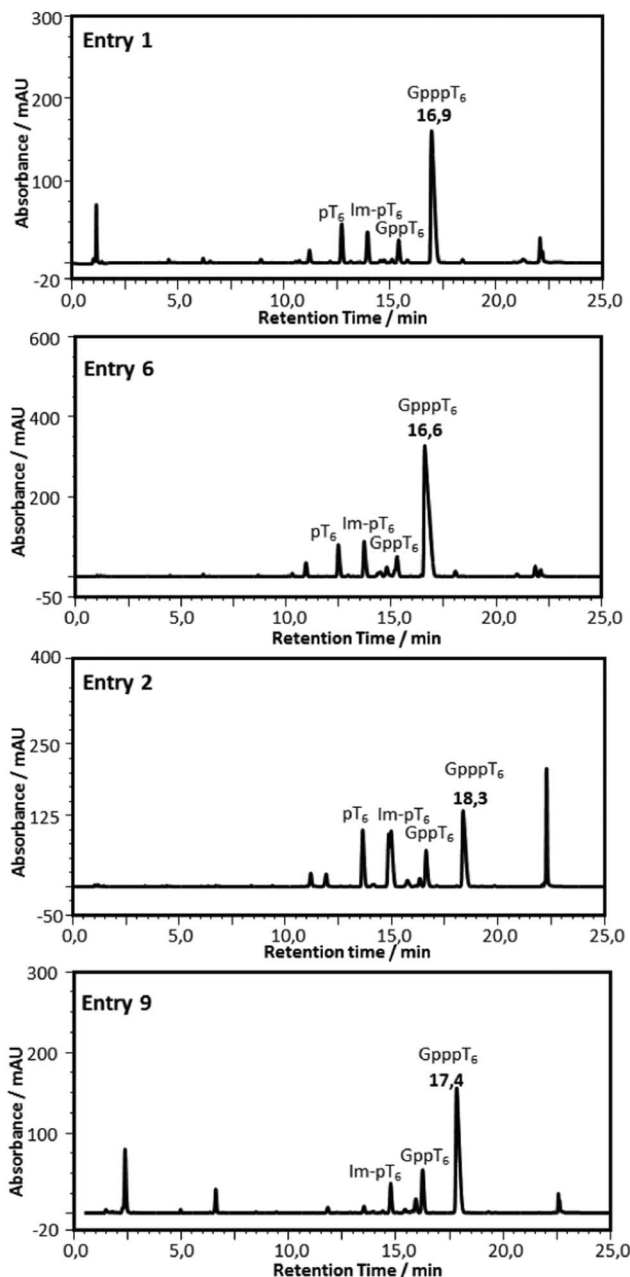


Fig. 3 Anion-exchange HPLC profiles of the crude mixture of 5'-GpppT₆ obtained with ZnCl₂ (Table 2, entry 1), *N. caerulescens* purified extract (fraction 3) (Table 2, entry 6), NiCl₂ (Table 2, entry 2) and with *P. douarrei* purified extract (Table 2, entry 9).

for RNA synthesis on solid support, is to use base-labile protecting groups exclusively removed under basic conditions without RNA damage. Thus, for GpppRNA synthesis (Table 2, entries 13, 14, 16, 17, 19, 20) RNA sequences were assembled on an automated synthesizer by the phosphoramidite solid-phase method involving the base-labile 2'-O-PivOM groups.²² As for T₆ sequences, after RNA elongation, the 5'-OH was converted into its phosphoroimidazolide **3** ready to react with GDP (Scheme 1) in the presence of purified catalysts derived from *N. caerulescens* and *P. douarrei* extracts. As shown for

Table 3 Comparison of the ratio of expected GpppT₆ and observed side-products GppT₆ depending on Ni composition of catalysts derived from Ni hyperaccumulating plants

Catalytic extracts	GpppT ₆ (%) ^a	GppT ₆ (%) ^a	Ni (ppm)
NiCl ₂	25	9	473 684
<i>P. douarrei</i> extract			
-crude	26	3	80 153
-purified	56	12	184 600
<i>G. pruinosa</i> extract			
-crude	18	3	73 863
-purified	42	15	150 395
<i>P. accuminata</i> extract			
- crude	33	13	115 163

^a Percentage yield of oligonucleotide in the crude as calculated from the integration of the IEX chromatogram.

GpppT₆ synthesis, the use of a clear solution resulting from centrifugation of catalytic extracts and GDP in DMF conditioned the success of the capping reaction. The purities of Gppp18-mers (Table 2, entry 16, 43%) (Fig. 5) and Gppp6-mers (Table 2, entry 17, 32%) obtained with *N. caerulescens* were similar to the one with conventional catalyst ZnCl₂ (entries 13 and 14, 46% and 45%). With *P. douarrei* extract, the coupling yields of the same RNA sequences (Table 2, entries 19 and 20) with GDP were reasonably lower: 34% and 23%, respectively.

Finally, we synthesized ^{7m}GpppAUA UUA (Table 2, entries 18 and 21) where as mentioned above the major difficulty is the fragility of N⁷-methylguanine under basic or acidic conditions. RNA sequences were assembled using 2'-O-propyloxymethyl (PrOM) ribonucleosides amidites.²³ This 2'-O-protecting group structurally closed to PivOM could be removed upon nucleophilic attack in dry organic solvent without using ammonia conditions which would lead to complete destruction of the N⁷-methylguanine structure. After elongation on synthesizer, both RNA 6-mers were functionalized at their 5'-end with a phosphoroimidazolide to react with ^{7m}GDP bis(tri-*n*-butylam-

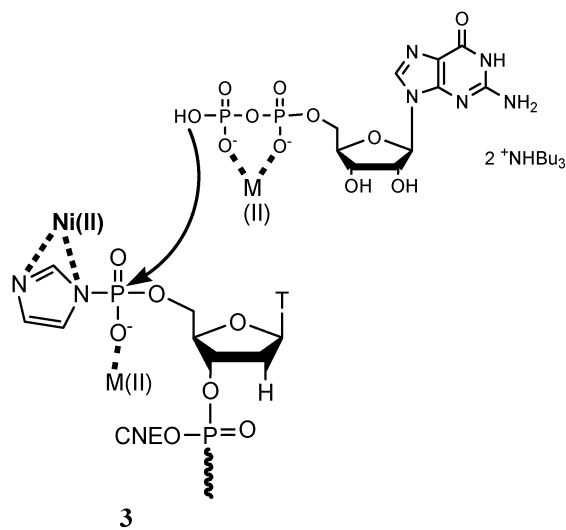


Fig. 4 Hypothetic mechanism for polymetallic participation in the coupling reaction.

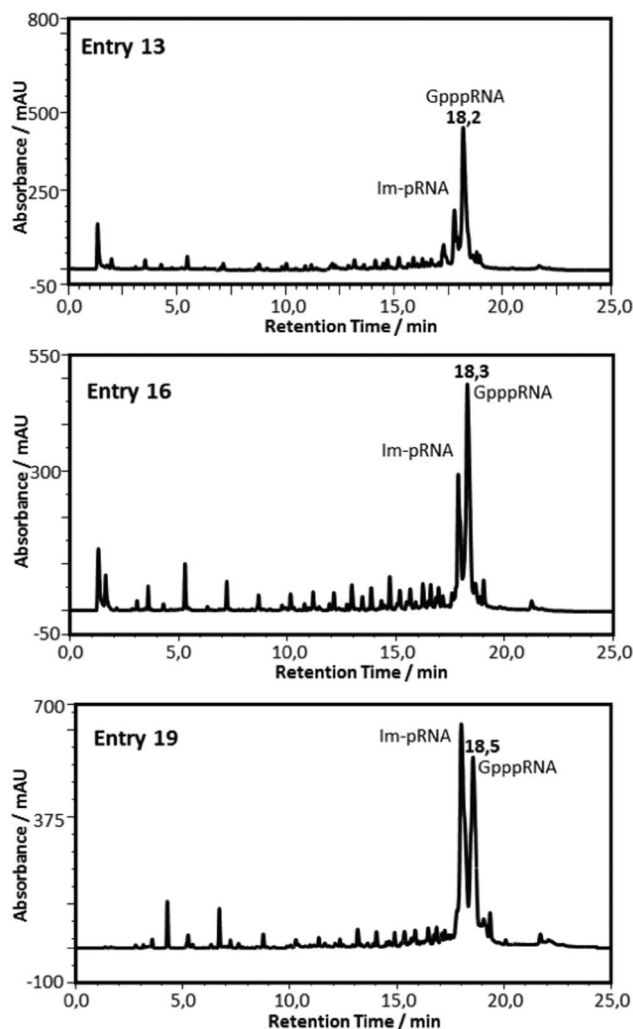


Fig. 5 Anion-exchange HPLC profiles of the crude mixture of 5'-GpppAGUUGUUAGUCUUACUGG obtained with ZnCl_2 (Table 2, entry 13) and 5'-GpppA_{OMe}GUUGUUAGUCUUACUGGA obtained with *N. caeruleus* purified extract (fraction 3) (Table 2, entry 16) and with *P. douarrei* purified extract (Table 2, entry 19).

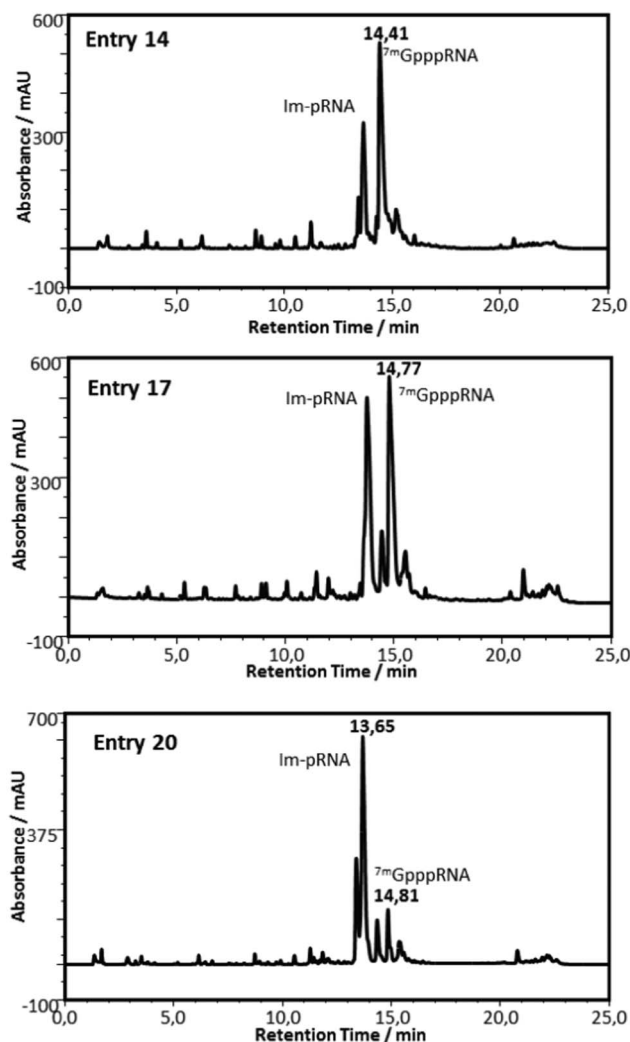


Fig. 6 Anion-exchange HPLC profiles of the crude mixture of 5'-7mGpppAUUUUA obtained with ZnCl_2 (Table 2, entry 14), with *N. caeruleus* purified extract (fraction 3) (Table 2, entry 17) and with *P. douarrei* purified extract (Table 2, entry 20).

monium) in the presence of ZnCl_2 (Table 2, entry 15), purified *N. caeruleus* (Table 2, entry 18) and *P. douarrei* extracts (Table 2, entry 21) (Scheme 1). First attempts in the same conditions previously used for GpppRNA were not successful and depurination of N^7 -methylguanosine was observed which was confirmed by the disappearance of the peak in the HPLC profile monitored at 300 nm (characteristic wavelength of UV absorption of ^7mG).²⁰ This major problem was certainly due to the presence of acidic traces in the catalytic extracts. When the acidity of catalyst derived from *N. caeruleus* (Table 2, entry 18) was neutralized by refluxing the catalytic extract in dioxane, the purity of $^7\text{mGpppAUUUUA}$ was acceptable (33%) and near to ZnCl_2 (38%) (Fig. 6). It should be underlined that this is the first efficient all-chemical synthesis on solid support of an RNA with a ^7m cap structure and free 2'-OH. Indeed only short 2'-OMe RNA bearing a ^7m cap were reported.^{18,24} Finally, with *P. douarrei* extract, the capping reaction was not as

efficient as with *N. caeruleus* since the desired compound was present at 7% only in the reaction mixture.

Experimental

Preparation of catalytic extracts from metallophytes species

Leaves were harvested before flowering, air-dried and crushed. The obtained solid (150 g) was calcined at 400 °C for 5 h and the resulting powder (148 g) was added to 1 L of a solution of 5 M HCl solution. The solution was heated at 60 °C and stirred for 2 h. The reaction mixture was filtered on celite. The resulting solutions, composed of different metal chlorides, were then concentrated under vacuum. Dry residues were either used crude or partially purified in order to decrease the concentration of alkali and alkaline earth metals in the catalytic solid. When Ni hyperaccumulating metallophytes (*P.*

douarrei and *G. pruinosa* and *P. accuminata*) were used, a chelating resin such Dowex M4195, allowed Ni concentration and partial elimination on undesired metals ions. The catalytic solution was introduced on the uppermost surface of the Dowex M4195 (about 60 g of resin per gram of solid). Operating purification conditions were as follows: elution of alkali and alkaline earth metals with HCl at pH 2.5 (3 mL min⁻¹); transition metals elution was performed with 12 M HCl. With Zn hyperaccumulating metallophytes (*N. caerulescens* and *A. vulneraria*), Amberlite IRA 400 ion exchange resin was used for adsorption of Zn^{II} on the resin, and elution of alkali and alkaline earth cations. Treatment with 0.5 M HCl eliminated a part of Fe^{III} fixed on the resin before the elution of heavy metals Zn^{II}, Cd^{II}, Pb^{II} with 0.005 M HCl.

ICP-MS was used to determine the composition of the various plant extracts obtained. ICP-MS analyses were performed using the Metal Analysis of total dissolved solutes in water. The samples were acidified with nitric acid 2.5% and stirred for 30 min. The digestates were diluted to 0.005 g L⁻¹. Three blanks are recorded for each step of the digestion and dilution procedure on a HR-ICP-MS Thermo Scientific Element XR.

Coupling of solid-supported T₆ or RNA phosphoroimidazolidate with GDP

Guanosine-5'-diphosphate sodium salt was converted before coupling into its tri-*n*-butylammonium salt as previously described.¹⁹ In a dry 2 mL microcentrifuge tube (Costar), bis(tri-*n*-butylammonium) GDP (103 mg, 0.14 mmol) and the freshly dehydrated catalytic extracts required for the coupling (0.2 mmol) whose amount was estimated from the concentration of the metal (Zn or Ni) determined by ICP-MS (Table 1) were mixed in anhydrous DMF (0.5 mL). The tube was closed and the mixture was vortexed for 5 min on a Top-Mix 1118 (Fischerbrand) and centrifuged in a tabletop centrifuge (Sigma 1-13) at 6000 min⁻¹ for 30 s. This operation was repeated twice. The supernatant was taken using a glass syringe filled with 3 beads of 4 Å molecular sieves. Using another syringe, the solution was applied to the column containing the solid-supported 5'-phosphoroimidazolidate oligonucleotide 3 (1 µmol), and left to react for 18 h at 30 °C. The solution was removed and the support was washed with water (2 × 2 mL), then with a 0.1 M aqueous solution of EDTA (pH 7, 2 × 2 mL), and dry CH₃CN (4 × 2 mL). Finally the column was dried by blowing argon through it during 1 min. The same procedure was applied for coupling with ^{7m}GDP except a two-fold decrease in the quantities of reagents used.

Conclusion

This study clearly shows the interest of the metallophyte-based polymetallic catalysts for the synthesis of RNA 5'-cap structures. They differ notably from classical systems which employ chemical uniformity and monometallic composition. Partial purification of the plant extracts was sufficient for catalytic performance. The polymetallic composition of our bio-based catalysts is particularly adapted to multi-step transformations,

which are founded on different mechanisms and so required different catalytic effects. The coupling of the cap structure on DNA or RNA is illustrative for numerous issues in the development of a new concept for the "ecological" concept. Interestingly in the development of new "environmental" chemical processes it is noteworthy that this pyrophosphate bond formation was also the key point of an atom-economic methodology recently developed with the use of a ball-mill.²⁵ Future developments of these plant-based catalysts are exciting, and can inspire the creativity of synthetic chemists. They constitute an important incentive for the development of phytoextraction and the subsequent polymetallic catalysis.

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