FINAL REPORT

Manufacture of TATB and TNT from Biosynthesized Phloroglucinols

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14. ABSTRACT

In route to the microbial synthesis of mono-O-methylphloroglucinols, phloroglucinol O-methyl transferase (POMT) from Rosa chinensis var. spontanea has been successfully de novo synthesized in codon-optimized form for expression in E. coli, which is the host currently used for microbial synthesis of phloroglucinol (PG) from glucose. The specific activity of heterologously-expressed, codon-optimized POMT is at 0.02 U/mg, which is 5-fold higher than the specific activity of POMT purified to homogeneity from Rosa chinensis var. spontanea petals. Similarly, orcinol O-methyl transferase (OOMT) protein sequence was identified from GenBank, codon optimized for E. coli expression using the DNA2.0 algorithm, and synthesized. Expressing this gene in E. coli gave a specific activity at 0.006 U/mg using crude lysate. Efforts had been made to create the first E. coli mono-O-methylphloroglucinol synthesizing construct. E. coli PG1/pKIT1.008 synthesized 0.7 g/L methoxyresorcinol in the medium under fed-batch fermentor-controlled conditions. A methionine supplementation strategy was developed to give a 40% increase in mono-O-methylphloroglucinol production under fed-batch fermentor-controlled conditions. It is believed that mono-O-methylphloroglucinol is toxic to E. coli cells and difficulties were experienced in maintaining a healthy culture in the fermentor. To circumvent this issue, resin-based extractive fermentation was carried out as an external loop throughout the run. By using a strong anion-exchange resin under optimized conditions, E. coli PG1/pKIT1.008 synthesized 2 g/L mono-O-methylphloroglucinol in 70 h after inoculation. Simultaneously, efforts had been made in both strain development and optimizing fermentation conditions for microbial phloroglucinol synthesis. Under optimized resin-based extractive fermentation, E. coli PG1/pKIT10.080 synthesized 25 g/L of phloroglucinol.

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Acknowledgements

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Abstract

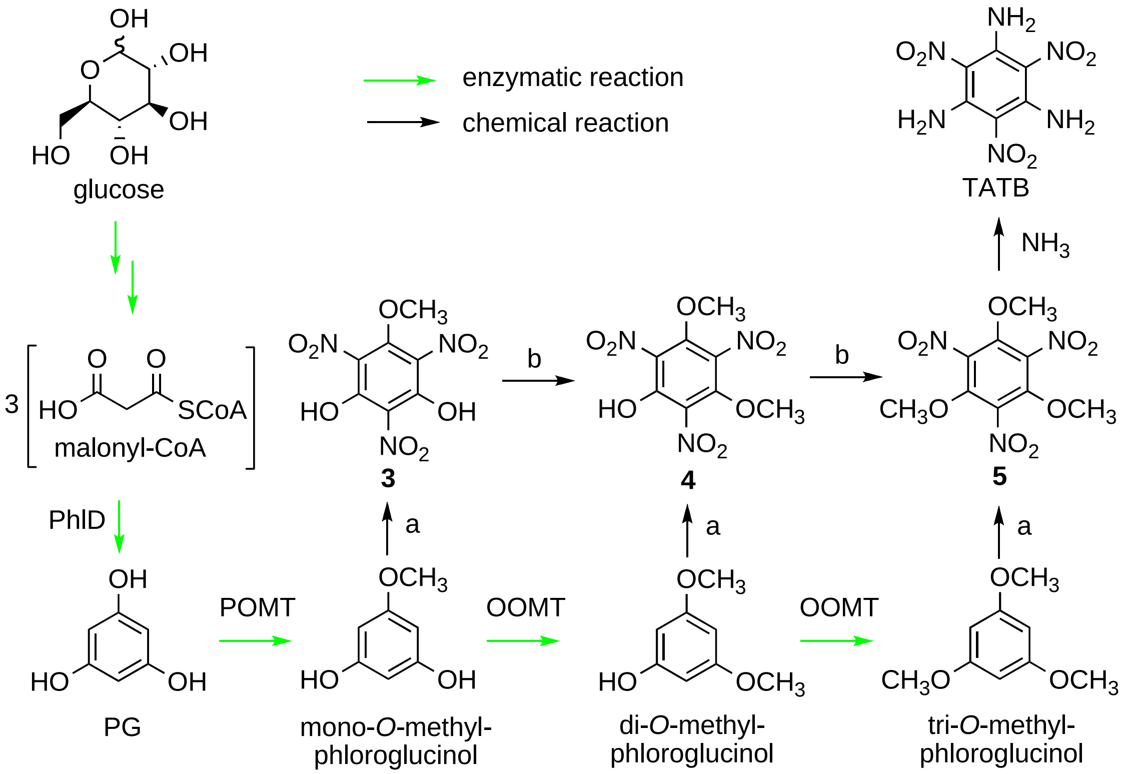
In route to the microbial synthesis of mono-O-methylphloroglucinols, phloroglucinol O-methyl transferase (POMT) from Rosa chinensis var. spontanea has been successfully de novo synthesized in codon-optimized form for expression in E. coli, which is the host currently used for microbial synthesis of phloroglucinol (PG) from glucose. The specific activity of heterologously-expressed, codon-optimized POMT is at 0.02 U/mg, which is 5-fold higher than the specific activity of POMT purified to homogeneity from Rosa chinensis var. spontanea petals. Similarly, orcinol O-methyl transferase (OOMT) protein sequence was identified from GenBank, codon optimized for E. coli expression using the DNA2.0 algorithm, and synthesized. Expressing this gene in E. coli gave a specific activity at 0.006 U/mg using crude lysate.

Efforts had been made to create the first E. coli mono-O-methylphloroglucinol synthesizing construct. E. coli PG1/pKIT1.008 synthesized 0.7 g/L methoxyresorcinol in the medium under fed-batch fermentor-controlled conditions. A methionine supplementation strategy was developed to give a 40% increase in mono-O-methylphloroglucinol production under fed-batch fermentor-controlled conditions. It is believed that mono-O-methylphloroglucinol is toxic to E. coli cells and difficulties were experienced in maintaining a healthy culture in the fermentor. To circumvent this issue, resin-based extractive fermentation was carried out as an external loop throughout the run. By using a strong anion-exchange resin under optimized conditions, E. coli PG1/pKIT1.008 synthesized 2 g/L mono-O-methylphloroglucinol in 70 h after inoculation. Simultaneously, efforts had been made in both strain development and optimizing fermentation conditions for microbial phloroglucinol synthesis. Under optimized resin-based extractive fermentation, E. coli PG1/pKIT10.080 synthesized 25 g/L of phloroglucinol.

Objective

Draths' approach to biosynthesized phloroglucinols begins with microbial conversion of glucose to malonyl-CoA (Scheme 1). Phloroglucinol synthase (PhlD, Scheme 1) then catalyzes the conversion of malonyl-CoA into phloroglucinol. Phloroglucinol O-methyl transferese (POMT, Scheme 1) then catalyzes the reaction of *S*-adenosylmethionine with phloroglucinol to form 5-methoxyresorcinol (mono-*O*-methylphloroglucinol). Orcinol *O*-methyl transferase (OOMT, Scheme 1) catalyzes the sequential methylations leading to 3,5-dimethoxyphenol (di-*O*-methylphloroglucinol), and 1,3,5-trimethoxybenzene (tri-*O*-methylphloroglucinol). The proposed biocatalytic route potentially eliminates byproduct waste streams by chemically synthesizing TATB from microbially synthesized *O*-methylphloroglucinol derivatives. It could also eliminate 'red water' waste streams by chemically synthesizing TNT from 2-methylphloroglucinol derived from microbially synthesized phloroglucinol or mono-*O*-methylphloroglucinol.

Scheme 1:



(a) H_2SO_4, HNO_3; (b) $HC(OCH_3)_3$

Background

The discovery[1] of *phlD*-encoded phloroglucinol synthase and the construction[1] of an *E. coli* strain capable of synthesizing PG from glucose resulted from the project "Biosynthesis of Thermally Stable Energetic Compounds" funded by the Office of Naval Research (ONR Award No: N000140210725; January 1, 2004 – April 30, 2008). This research was carried out in the Frost group at Michigan State University.

Examination of PG biosynthesis in *Pseudomonas fluorescens* and scale-up of PG biosynthesis in *E. coli* to 25 L is the focus of the project "Green Synthesis of Phloroglucinol: Exploiting *Pseudomonas fluroescens* and Scale-up" funded by the Office of Naval Research (ONR Award No: N000140710076; October 4, 2006 – October 14, 2009). This research was carried out at Draths Corporation.

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[1] Achkar, J.; Xian, M.; Zhao, H.; Frost, J. W. *J. Am. Chem. Soc.* **2005**, *127*, 5332.

Materials and Methods

Efforts directed towards obtaining the gene encoding POMT first focused on the possibility of initially purifying POMT from petals of *Rosa chinensis* var. *spontanea*. Attention then turned to the protein sequence that has been published for POMT. Optimized POMT gene was obtained using the program Gene Designer (DNA2.0), and synthesized by DNA2.0. Similar approach was used to identified and express OOMT activity in *E. coli*. Expression of POMT in the benchmark phloroglucinol synthesizing *E. coli* PG1/pKIT10.080 created the first mono-*O*-methylphloroglucinol synthesizing construct. The impact of *S*-adenosylmethionine availability was evaluated on the synthesis of mono-*O*-methylphloroglucinol under fermentor-controlled conditions. Resin-based extractive fermentation was used to inline removal of toxic mono-*O*-methylphloroglucinol from the medium. Coupling metabolic engineering and reaction engineering, improved phloroglucinol synthesizing *E. coli* PG1/pKIT10.080 was evaluated under resin-based extractive fermentor-controlled conditions.

The current production of TATB relies on the preliminary deprotonation with sodium hydroxide of at least one of the hydroxyl groups of PG to render it soluble in water. The resulting sodium phloroglucinate is then subjected to nitrosation with sodium nitrite and nitric acid followed by *N*-oxidation to generate 2,4,6-trinitrophloroglucinol (**1**, Scheme 1). This compound is then *O*-alkylated with a trialkylorthoformate in organic solvent. Finally TATB is generated by amination with ammonia gas and concomitant displacement of the corresponding alcohol. In order to reduce or altogether eliminate the use of trialkylorthoformates, we proposed to use various degrees of *O*-methylated phloroglucinol derivatives from biosynthetic sources as starting materials, namely 5-methoxyresorcinol, 3,5-dimethoxyphenol, and 1,3,5-trimethoxybenzene.

The first challenge that needed to be addressed in these alternative syntheses was the feasibility of tri-nitrating the aromatic ring. It was not immediately obvious that adding three nitro groups to the *O*-methylated derivatives would be possible. The primary concern was the fact that alkoxy substituents are less activating than hydroxyl substituents in electrophilic aromatic substitution reactions. Accordingly, it would hold true that the nitration of the proposed new starting materials would get progressively more difficult with the increase in number of methoxy groups in place of hydroxyl groups on PG. Additionally, complete displacement of hydroxyl groups for methoxy substituents, as in 1,3,5-trimethoxybenzene, eliminates the option of solubilizing the material via preliminary deprotonation. Therefore, we set out to investigate various nitration strategies in an attempt to identify one that could be used to successfully tri-nitrate all three *O*-methylated phloroglucinol derivatives.

A cursory review of the literature indicates the tri-nitration of activated benzene derivatives is difficult, presumably because of the deactivating effect experienced by the ring from the addition of the first two nitro groups. Di-nitration appears to be rather facile, but the harsh conditions required to add a third nitro group to the aromatic ring tend to compromise yields as a result of oxidative side reactions. The currently employed nitration process of nitrosation followed by oxidation en route to TATB gives good yields so it was the first approach tried at ATK Launch Systems with the *O*-methylated phloroglucinols. As it turns out, the nitrosation/oxidation method proved insufficient for tri-nitrating the *O*-methylated phloroglucinol derivatives and, after much experimentation, the more traditional mixed acid nitration conditions (H_2SO_4, HNO_3) yielded the best results.

Efforts directed towards obtaining the gene encoding POMT first focused on the possibility of initially purifying POMT from petals of *Rosa chinensis* var. *spontanea*. Approximately 60 g of petals from *Rosa chinensis* var. *spontanea* were obtained with the cooperation of Quarryhill Botanical Garden in Glen Ellen, California. *Rosa chinensis* is the “wild” rose species native to China that biosynthesizes tri-*O*-methylphloroglucinol and from which the gene encoding POMT has been isolated. Because *Rosa chinensis* has only a limited flowering period (mid-March to mid-April), petals were collected by Quarryhill Botanical Garden’s staff, quick-frozen in liquid nitrogen and then shipped to Draths on dry ice. From 10 g of petals, we obtained 12 mg of protein. However, derivatization followed by analysis by gas chromatography using a capillary column interfaced with a flame ionization detector failed to detect 5-methoxyresorcinol (mono-*O*-methylphloroglucinol) when petal extract was incubated with *S*-adenosylmethionine.

Attention then turned to the sequence that has been published[2] for POMT and de novo synthesis of the *POMT* gene. Because the published[2] enzyme activity for POMT purified to homogeneity was so low, codon optimization of the synthesized *Rosa chinensis* gene for heterologous expression in *E. coli* was pursued. Optimization of codon usage for expression of *Rosa chinensis* var. *spontanea POMT* in *E. coli* is summarized in Table 5 (Appendices I and II).

An attractive manufacturing route to TATB from phlorolgucinol has been developed by ATK Launch Systems. A key step in this manufacturing route is the etherification of 2,4,6-trinitrophloroglucinol using triethylorthoformate to produce 1,3,5-triethoxy-2,4,6-trinitrophloroglucinol. This etherification step generates 640 kg of mixed, flammable solvents (ethanol, diethyl ether, and ethyl formate) as waste byproducts for every 180 kg of TATB produced. ATK has also employed trimethylorthoforate in the etherifcation step with similar results.

Draths Corporation has targeted microbial synthesis of phloroglucinol from glucose for commercial development. The scientific question to be explored with SERDP funding is whether 5-methoxyresorcinol (mono-*O*-methylphloroglucinol), 3,5-dimethoxyphenol (di-*O*-methylphloroglucinol, and 1,3,5-trimethoxybenzene (tri-*O*-methylphloroglucinol) can be microbial synthesized and chemically nitrated so that the concentrations of triethylorthoformate and trimethylothorformate used in the current etherification step (Scheme 1) can either be reduced or completely eliminated.

Draths Corporation established methodology for the chemical synthesis of 5-methoxyresorcinol (mono-*O*-methylphloroglucinol) and 3,5-dimethoxyphenol (di-*O*-methylphloroglucinol) involving the reaction of methanol with PG. ATK Launch Systems then examined the nitration of these methylated phloroglucinols in addition to commercially obtained 1,3,5-trimethoxybenzene (tri-*O*-methylphloroglucinol). The partially methylated trinitrophenols were

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[2] Wu, S.; Watanabe, N.; Mita, S.; Dohra, H.; Ueda, Y.; Shibuya, M.; Ebizuka, Y. *Plant Physiol* **2004**, *135*, 95.

then converted upon reaction with trimethylorthoformate to the same 1,3,5-trimethoxy-2,4,6-trinitrobenzene obtained from nitration of 1,3,5-trimethoxybenzene (tri-*O*-methylphloroglucinol). The results of these studies suggest that the complete elimination or at least substantial reduction in quantities of trimethyl- or triethylformate used during TATB manufacture is possible. As a critical first step to microbial synthesis of methylated phloroglucinols, Draths Corporation has successfully synthesized the codon-optimized gene encoding phloroglucinol *O*-methyltransferase (POMT) found in *Rosa chinensis* var. *spontanea* (Chinese rose) petals and heterologously expressed the codon-optimized *POMT* gene in *Escherichia coli*.

TNT is used extensively in bomb fill due to its relatively low melting point and processing ease. Current production requirements call for a minimum of 1,800,000 kg/year of virgin TNT for various munitions applications. It is manufactured in the United States at the Radford Army Ammunition Plant from a mixed acids nitration process that has changed very little in over 60 years. This production method provides a quick and inexpensive route to TNT but is burdened with an environmentally problematic waste stream. The so-called "red water" waste from TNT production is a result of the treatment of the TNT process stream with sellite (sodium sulfite) to remove incompletely nitrated nitrotoluenes and off-isomers of nitrotoluenes from the TNT. This TNT red water is a regulated waste, and while much has been done to remediate this waste, it remains a practical and environmental concern.

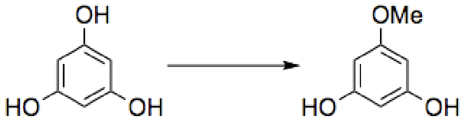
Draths Corporation explored a new synthesis method of 2,4,6-trinitrotoluene (TNT) based on 2-methylphloroglucinol. Conversion of 2-methylphloroglucinol to TNT did not involve nitration reactions and does not generate "red water" waste. The source of 2-methylphloroglucinol will either be chemical isomerization of microbe-synthesized 5-methoxyresorcinol (mono-*O*-methylphloroglucinol) or chemical methylation of microbe-synthesized PG. Research directed towards development of a new synthesis of TNT that avoids "red water" waste is slated for the upcoming second year of SERDP funding.

Results and Discussion

Biological investigation

Approximately 60 g of petals from *Rosa chinensis* var. *spontanea* were obtained with the cooperation of Quarryhill Botanical Garden in Glen Ellen, California. *Rosa chinensis* is the "wild" rose species native to China that biosynthesizes tri-*O*-methylphloroglucinol and from which the gene encoding POMT has been isolated. Because *Rosa chinensis* has only a limited flowering period (mid-March to mid-April), petals were collected by Quarryhill Botanical Garden's staff, quick-frozen in liquid nitrogen and then shipped to Draths on dry ice. From 10 g of petals, we obtained 12 mg of protein. However, derivatization followed by analysis by gas chromatography using a capillary column interfaced with a flame ionization detector failed to detect mono-*O*-methylphloroglucinol when petal extract was incubated with *S*-adenosylmethionine. Because the published enzyme activity for POMT purified to homogeneity was so low, codon optimization of the synthesized *Rosa chinensis* gene for heterologous expression in *E. coli* was pursued.

De novo synthesized, codon-optimized POMT was inserted into pET22B. This plasmid insert is expressed from a T7 promoter and has a C-terminal his-tag, which will assist with purification of POMT to homogeneity in the future. After transformation, the resulting *E. coli* BL21(DE3)/pET22B containing the plasmid-localized, codon-optimized POMT insert was cultured and the cells harvested, lysed in a French press. After centrifugation, the clarified lysate had a specific activity for POMT of 0.02 U/mg (Table 1). This compares with 0.004 U/mg for POMT purified to homogeneity from *Rosa chinensis* var. *spontanea* petals. POMT activity was assayed with minor modification as described by Wu, S. in literature (Figure 1). Phloroglucinol (2 mM), dithiothreitol (10 mM), *S*-adenosylmethionine (5 mM), glycerol (10 mM) was incubated in 50 mM sodium phosphate buffer at pH 7 at rt. The assay was initiated by the addition of crude lysate of *E. coli* construct BL21(DE3)/pPOMT. A total volume of 5 mL reaction mixture was setup. A 0.9 mL sample was taken at 10, 20 and 30 min and quenched with 0.1 mL trichloroacetic acid (10%). The sample was centrifuged and the supernatant was dried under reduced pressure. The synthesized mono-*O*-methylphloroglucinol was derivatized with BSTFA and quantified using GC. One unit of mono-*O*-methylphloroglucinol forming activity was defined as the catalyzed formation of 1 μmol mono-*O*-methylphloroglucinol per min at rt.



Reaction condition: dithiothreitol (10 mM)
glycerol (10%)
S-adenosyl-methionine chloride (5 mM)
phloroglucinol (2 mM)
crude lysate of BL21(DE3)/pET22b-POMT
in sodium phosphate buffer (50 mM, pH 7)

Specific activity: 0.02 U/mg

Figure 1. POMT activity assay

Figure 2 shows a GC spectral trace for the formation of mono-*O*-methylphloroglucinol. Phloroglucinol eluted off the column at 8.6 min retention time in the sample taken at t=0. A new peak shows up at 8.1 min at t=10 min. The intensity of this new peak is increasing at t=20 and 30 min with the decrease of phloroglucinol in the sample. Finally, phloroglucinol is completely disappeared in the overnight sample and this new peak at 8.1 min is confirmed to be mono-*O*-methylphloroglucinol. A control construct without POMT gene was constructed as BL21(DE3)/pET22b. Incubating the reaction mixture previously discussed with this control crude lysate resulted in no mono-*O*-methylphloroglucinol formation. Coinjection of authentic mono-*O*-methylphloroglucinol with the POMT overnight reaction resulted in a perfect peak overlap at 8.1 min (Figure 3). This would provide a second line of evidence to the observed mono-*O*-methylphloroglucinol synthesizing ability using codon optimized POMT.

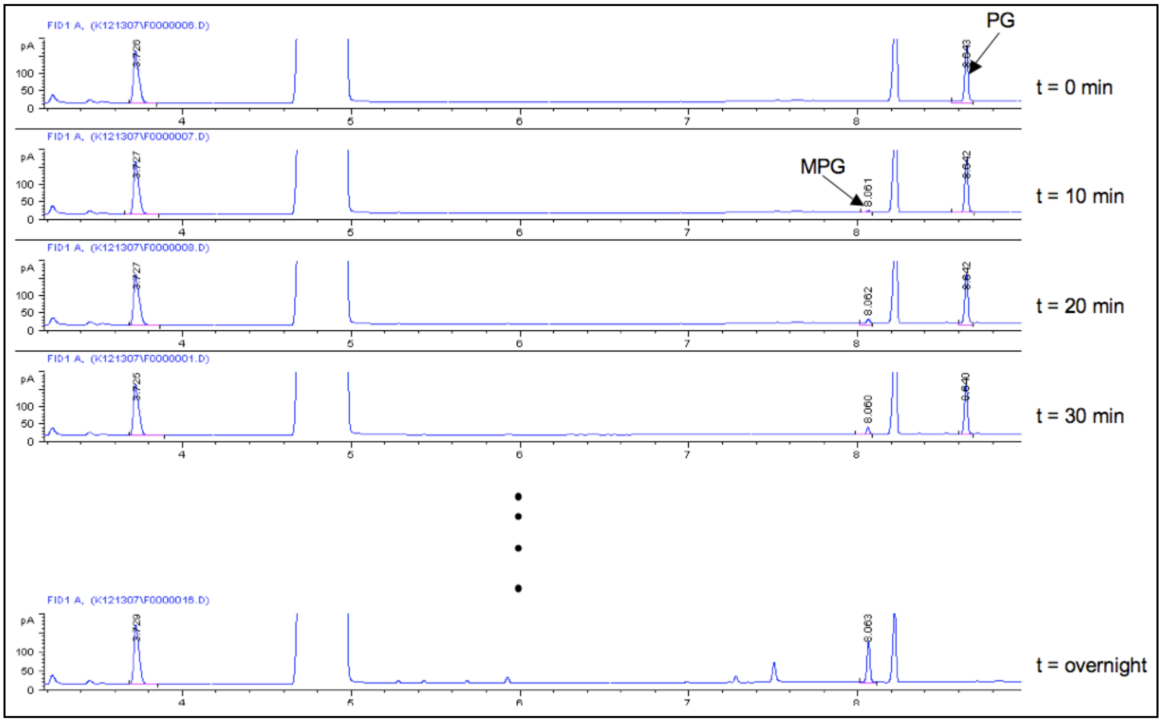


Figure 2. Spectral trace for mono-*O*-methylphloroglucinol formation.

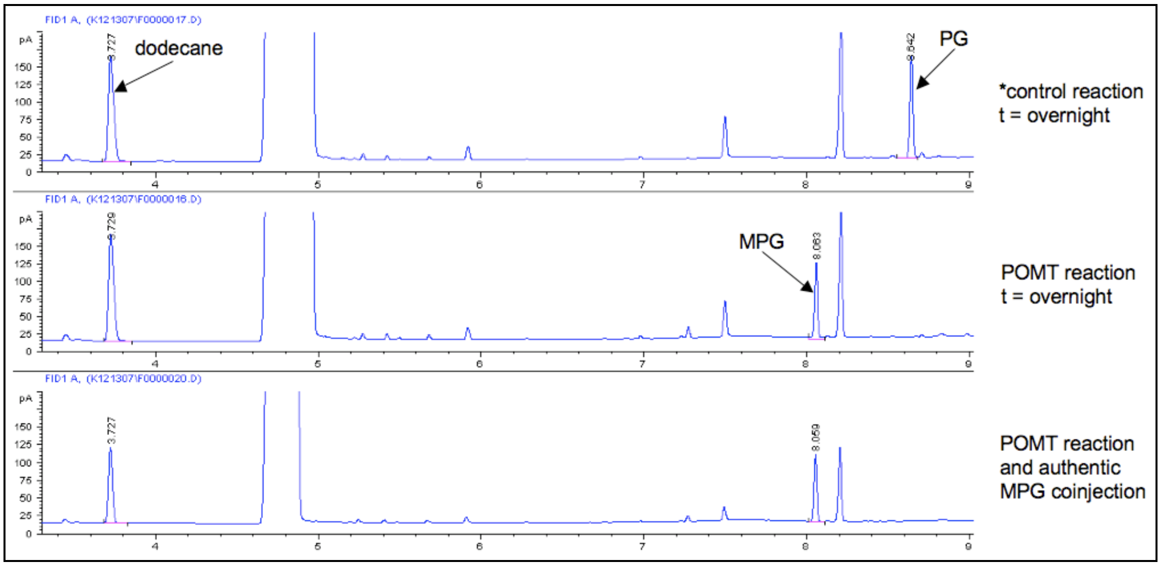
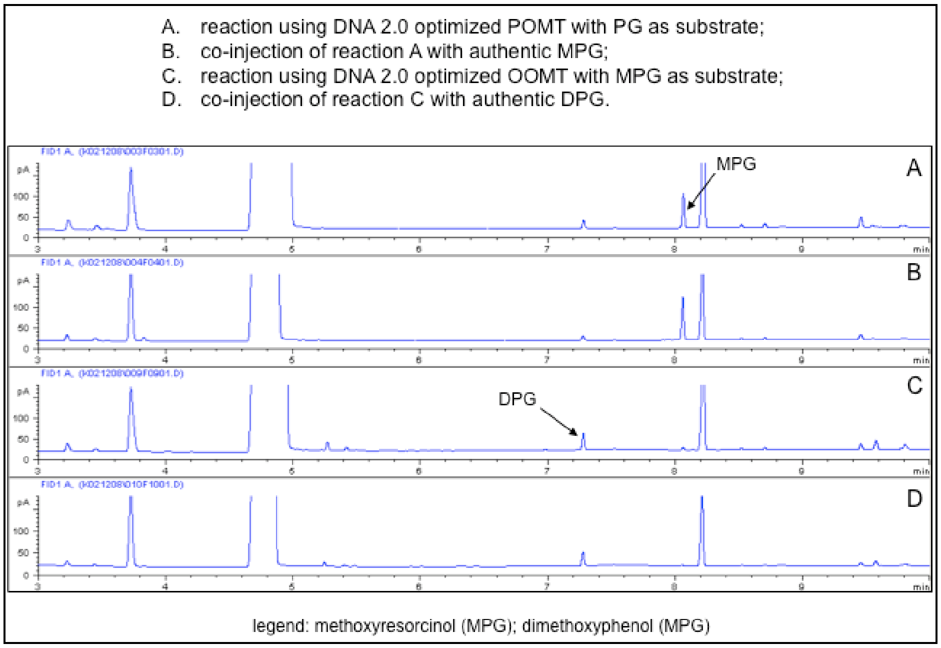


Figure 3. GC Spectra for control and authentic mono-*O*-methylphloroglucinol coinjection.

Draths Corporation had expressed active OOMT1 gene encoding orcinol *O*-methyltransferases in *E. coli* (Figure 1). Protein sequence of orcinol *O*-methyltransferase from *Rosa hybrida* was codon optimized and back-translated into DNA sequence for *E. coli* expression using Gene Designer (DNA2.0). The resulting OOMT1 gene was synthesized and cloned into expression vector pET22b (Novagen) under a T7 promoter. The resulting plasmid pOOMT1 was transformed into *E. coli* BL21(DE3) and was assayed for methylase activity using mono-*O*-methylphloroglucinol as substrate. The crude extract specific activity of this construct was found to be 0.006 U/mg (Table 1). The production of di-*O*-methylphloroglucinol in the reaction mixture was characterized by GC/FID and GC/MS (Figure 4).

Table 1. Specific activity of cloned *O*-methylases.

entry	Candidate	protein	substrate	specific activity (U/mg)
1	BL21(DE3)/pET22b		phloroglucinol	0
2	BL21(DE3)/pPOMT	POMT	phloroglucinol	0.02
3	BL21(DE3)/pOOMT1	OOMT1	phloroglucinol	n.d.
4	BL21(DE3)/pET22b		mono-*O*-methylphloroglucinol	0
5	BL21(DE3)/pPOMT	POMT	mono-*O*-methylphloroglucinol	n.d.
6	BL21(DE3)/pOOMT1	OOMT1	mono-*O*-methylphloroglucinol	0.006



A. reaction using DNA 2.0 optimized POMT with PG as substrate;
B. co-injection of reaction A with authentic MPG;
C. reaction using DNA 2.0 optimized OOMT with MPG as substrate;
D. co-injection of reaction C with authentic DPG.

legend: methoxyresorcinol (MPG); dimethoxyphenol (MPG)

Figure 4. GC analysis of OOMT enzyme reaction mixture.

Efforts had been made to create a single *E. coli* microbe that can synthesize mono-*O*-methylphloroglucinol from glucose. DNA2.0 optimized phloroglucinol *O*-methyltransferase gene (POMT) was excised from plasmid pET22b-pPOMT using *Nde*I and *Xho*I restriction enzymes. The resulting 0.7 kb gene was ligated with pKIT10.080, which had been previously treated with *Nde*I, *Xho*I and CIAP to afford pKIT1.007. A 1.6 kb *serA* locus was excised from plasmid pRC1.55B by digestion with *Sma*I and ligated to the plasmid pKIT1.007, which had been previously treated with *Sca*I and CIAP. The ligation mixture was transformed into *E. coli* PG1. Transformants carrying the *serA* insert were selected on M9 medium plates. In the resulting construct *E. coli* PG1/pKIT1.008, the *serA*, O_POMT and *phlD** genes are transcribed in the same direction. Cultivation of PG1/pKIT1.008 under fermentor controlled conditions synthesized 0.7 g/L mono-*O*-methylphloroglucinol. GC-MSD was used to characterize medium samples taken during fermentation and the corresponding total-ion chromatograms are shown in Figure 5. It is clear that there is an extra peak eluted from the column at 7.4 min retention time. From the mass spectrum of this peak (Figure 6), the molecular ion is believed to be at 284 m/z, which is the molecular weight of mono-*O*-methylphloroglucinol. The peak at 269 m/z indicates the loss of a methyl group from the molecular ion. Together with the GC-FID coinjection result with the authentic sample, we believe that *E. coli* PG1/pKIT1.008 synthesizes mono-*O*-methylphloroglucinol.

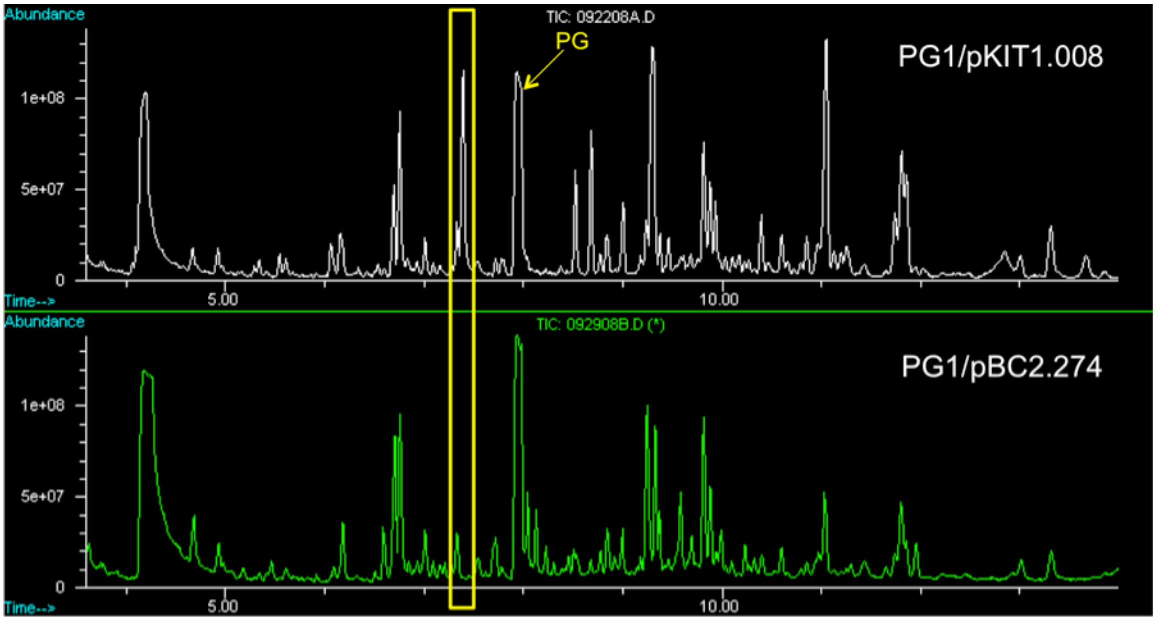


Figure 5. Total-ion chromatograms of fermentation samples taken from PG1/pKIT1.008 (upper) and PG1/pBC2.274 (control construct without POMT gene, below)

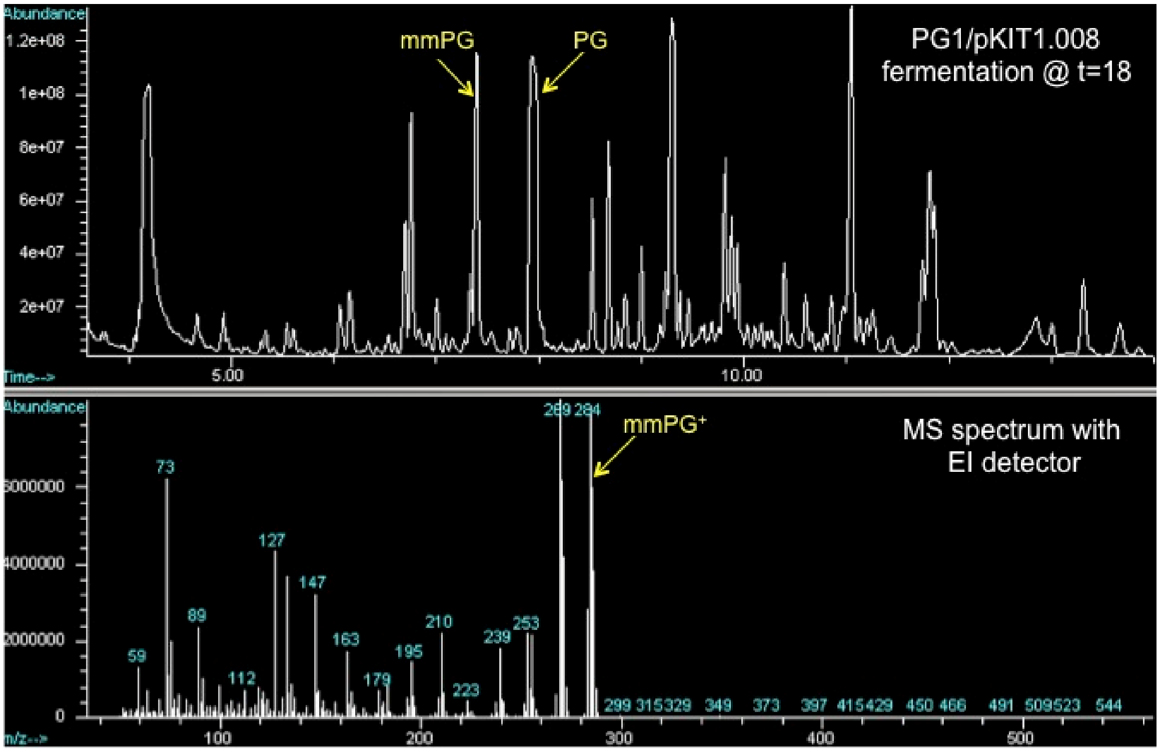


Figure 6. Mass spectrum of mono-*O*-methylphloroglucinol using EI detector.

Supplementation of the fermentation medium with methionine was found out to have a positive effect in mono-*O*-methylphloroglucinol production using PG1/pKIT1.008. The bar chart on the left side in Figure 7 shows a control fermentation of PG1/pKIT1.008 without methionine supplementation. The one on the right side shows a fermentation of PG1/pKIT1.008 with 0.4 g/L methionine supplemented at the time when IPTG was added to induce protein production. A similar production of mono-*O*-methylphlorglucinol was observed when compared to the control run. However, a trend of higher POMT activity was observed in the activity time-course, as shown in Figure 8. Further experiments were done in developing a methionine supplementation strategy. Figure 9 shows an optimized fed batch fermentation of PG1/pKIT1.008 under glucose-limited conditions. Under this optimized cultivation conditions, 0.2 g/L methionine was added to the culture every 6 h after protein production induced by adding IPTG. A 40% increase in mono-*O*-methylphloroglucinol production was observed after 44 h cultivation at a concentration of 1.1 g/L.

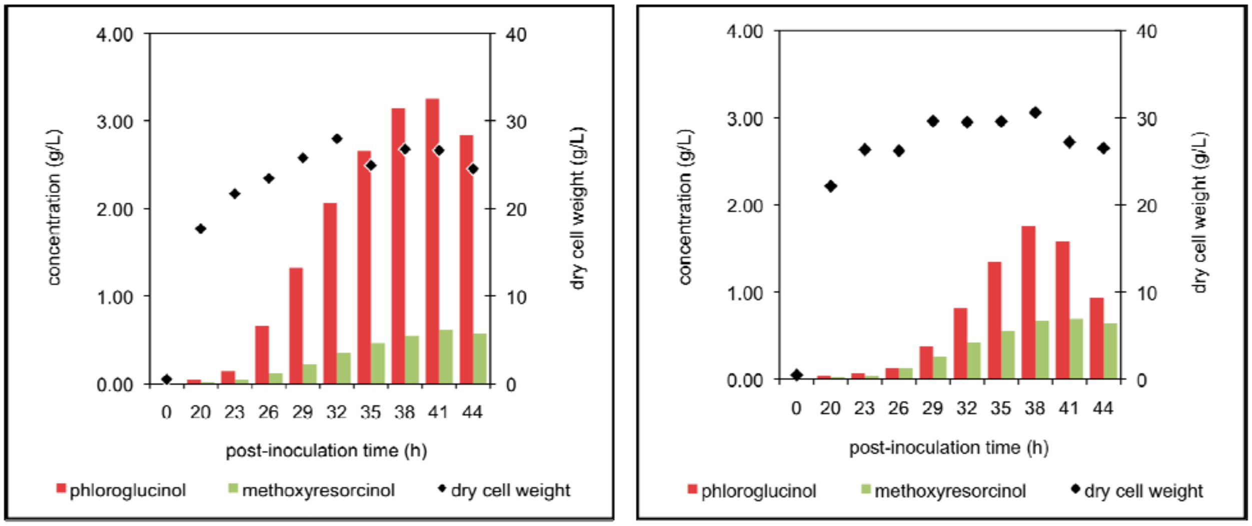


Figure 7. Fed-batch fermentation runs of *E. coli* PG1/pKIT1.008. (left) medium minus methionine, peak phloroglucinol titer at 41 h = 3.3 g/L, peak mono-*O*-methylphloroglucinol titer at 41 h = 0.6 g/L; (right) medium with 0.4 g/L methionine added at induction, peak phloroglucinol titer at 38 h = 1.8 g/L, peak mono-*O*-methylphloroglucinol titer at 41 h = 0.7 g/L.

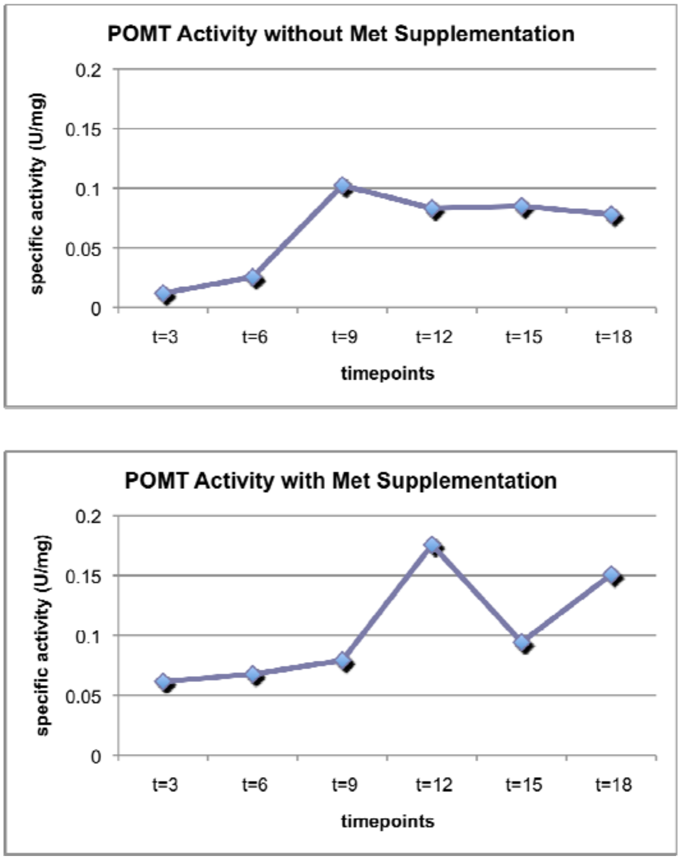


Figure 8. POMT enzyme activity traces for fed-batch *E. coli* PG1/pKIT1.008 fermentation runs.

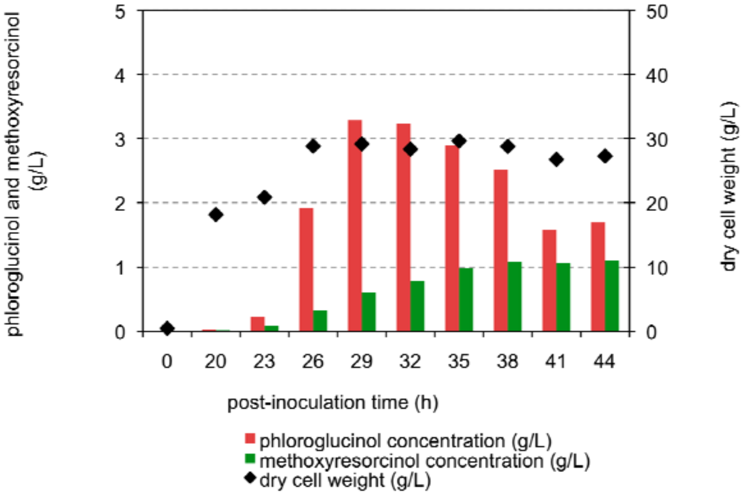


Figure 9. Optimized Fed-batch fermentation runs of *E. coli* PG1/pKIT1.008. Methionine (0.2 g/L) was added every 6 h after IPTG induction. Peak phloroglucinol titer at 29 h = 3.3 g/L; Final phloroglucinol titer at 44 h = 1.7 g/L; Peak mono-*O*-methylphloroglucinol titer at 44 h = 1.1 g/L; Final mono-*O*-methylphloroglucinol titer at 44 h = 1.1 g/L.

It is believed that mono-*O*-methylphloroglucinol is toxic to *E. coli* cells and we experienced difficulties in maintaining the culture in the fermentor. To circumvent this issue, attempts had been made to evaluate this microbe using resin-based extractive fermentation (Figure 10). By coupling our methionine feeding strategy and a strong anion-exchange resin column, *E. coli* PG1/pKIT1.008 synthesized 2 g/L mono-*O*-methylphloroglucinol in 70 h after inoculation (Figure 11), which accounts to another 45% increase in mono-*O*-methylphloroglucinol production.

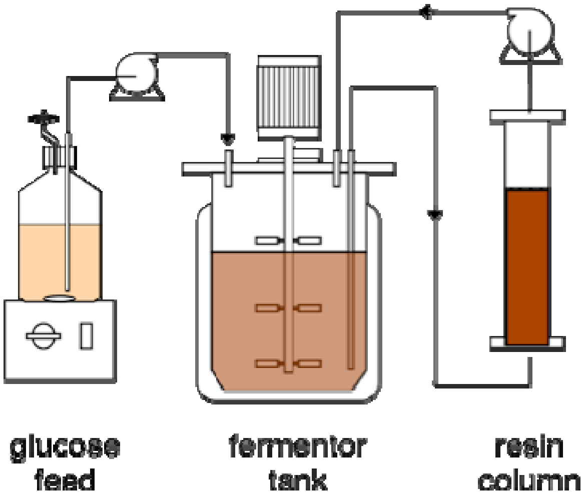


Figure 10. Diagram for resin-based extractive fermentation.

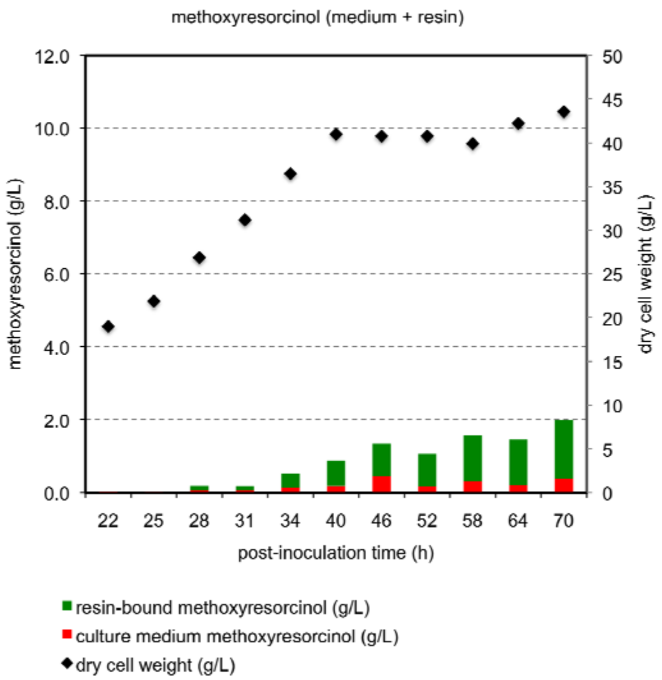


Figure 11. Resin-based extractive fermentation of *E. coli* PG1/pKIT1.008.

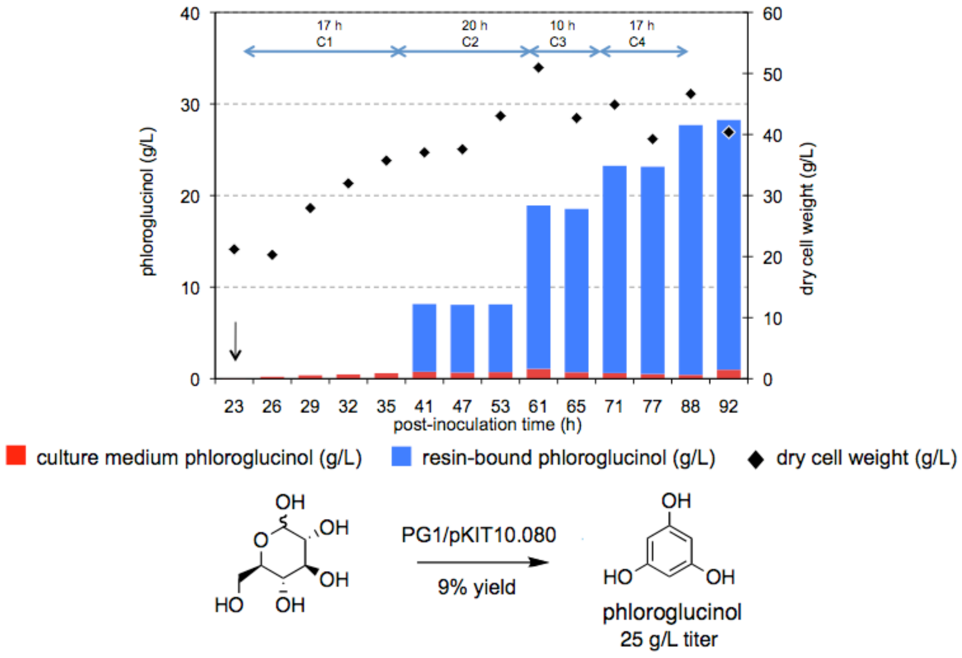


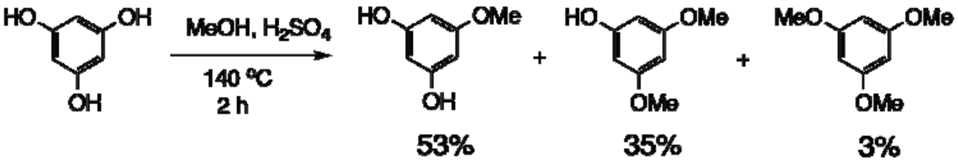
Figure 12. Resin-based extractive fermentation of *E. coli* PG1/pKIT10.080.

It has always been the top priority to improve the microbial synthesis of phloroglucinol. A series of genetically engineered *E. coli* was developed accordingly. Among these candidates, *E. coli* PG1/pKIT10.080 demonstrated a significant improvement in strain stability and phloroglucinol production under fed-batch fermentor-controlled cultivation. Previously, resin based extractive fermentation was employed inline to remove toxic substances generated during bacterial culturing. This approach was employed again to remove toxic phloroglucinol from the phloroglucinol production tank. Under optimized extractive fermentation conditions, *E. coli* PG1/pKIT10.080 synthesized 25 g/L phloroglucinol in 92 h at a yield of 9% (mol/mol) based on glucose consumed (Figure 12).

Chemical synthesis of TNT derivatives from phloroglucinol

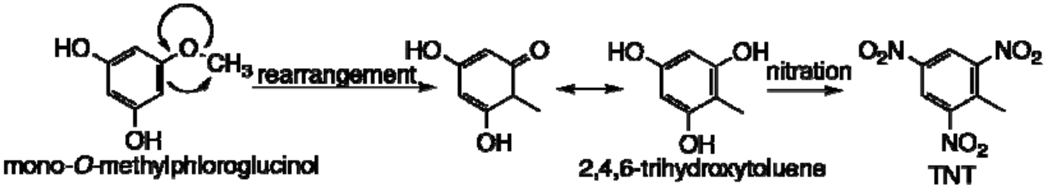
In conjunction with the effort to synthesize phloroglucinol biologically, attempts were also made to synthesize *O*-methylated and *C*-methylated phloroglucinol to be utilized as intermediates for synthesis of TNT.

The synthesis of mono-*O*-methylphloroglucinol, di-*O*-methylphloroglucinol, and tri-*O*-methylphloroglucinol, have been achieved with high yields by reacting phloroglucinol with methanol for 2 h at 140ºC in a sealed-tube with an aid of an acid catalyst such as H_2SO_4. The process provided a greater than 90% of O-methylated phloroglucinol with 53% of mono-O-methylphloroglucinol, 35% of di-O-methylphloroglucinol, and 3% of tri-O-methylphloroglucinol as summarized in Scheme 2.



Scheme 2. Synthesis of mono-O-methylphloroglucinol, di-O-methylphloroglucinol, and tri-O-methylphloroglucinol.

The synthesis of O-methylated phloroglucinol is an important part of our synthetic effort to develop an environmental friendly approach to the synthesis of TNT. Mono-O-methylphloroglucinol, for instance, could be rearranged to 2,4,6-trihydroxytoluene as an advanced intermediate that could be readily converted to 2,4,6-trinitrotoluene (TNT), scheme 3.



Scheme 3. Methyl migration of mono-O-methylphloroglucinol to 2,4,6-trihydroxytoluene as an approach to the synthesis of TNT.

This kind of rearrangement has literature precedent and has been studied extensively with aromatic substrate, such as anisole. In our effort to find potential catalysts for rearrangement of mono-O-methylphloroglucinol, anisole was used as model substrate to test against a series of metal oxide catalysts. Table 2 summarizes the results obtained for this work.

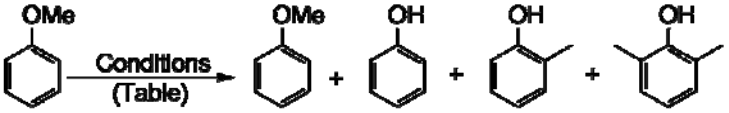


Table 2. Rearrangement of anisole over metal oxide catalysts.

Entry	Catalyst[b]	Anisole (g)	Hexanes (mL)	Temp (°C)	Time (h)	Results (% yields)[a] Anisole	Phenol	o-Cresol	Xylenol
1	Al_2O_3	1	5	140	4	56	8	5	3
2	Cr_2O_3	1	5	140	4	100	-	-	-
3	SiO_2	1	5	140	4	100	-	-	-
4	Co_3O_4	1	5	140	4	100	-	-	-
5	Y_2O_3	1	5	140	4	100	-	-	-
6	ZnO	1	5	140	4	100	-	-	-

[a]-GC yields; [b]-Catalyst were heat-activated at 600°C for 4h

Of the catalysts tested, Al_2O_3 (Table 2, entry 1) was the only one capable of rearranging anisole to *C*-alkylated products, o-cresol and xylenol, making it a potential catalyst for rearrangement of mono-O-methylphloroglucinol. Other catalysts, entries 2-6, did not provide any rearrangement products, however, phloroglucinol is relatively more reactive than anisole, catalysts for entries 2-6 could potentially be utilized for rearrangement of mono-O-methylphloroglucinol to 2,4,6-trihydroxytoluene.

Table 3 summarizes the overall result for our effort in attempting to rearrange mono-O-methylphloroglucinol to 2,4,6-trihydroxytoluene with a series of metal oxide catalyst and H_2SO_4. Overall, none of the catalyst tested provided any 2,4,6-trihydroxytoluene as rearranged product from mono-O-methylphloroglucinol (Table 3, entries 1-17).

Table 3. Attempt on rearrangement of mono-O-methylphloroglucinol to 2-methylphloroglucinol over metal oxide and sulfuric acid.

Entry	Reaction conditions Catalyst	Equi.	Solvent	Conc. [mg/mL]	Temp °C	Time (h)	Results
1	-	-	THF	10	90	4	No reaction
2	H_2SO_4	-	H_2SO_4	10	90	0.08	Charred
3	H_2SO_4	1	THF	10	100	4	No reaction
4	H_2SO_4	10	THF	10	120	6	Starting material + baseline material
5	Al_2O_3	2	THF	50	90	6	No reaction
6	Al_2O_3	2	THF	50	120	6	No reaction
7	Cr_2O_3	2	THF	50	120	6	No reaction
8	Y_2O_3	2	THF	50	140	2	No reaction
9	Ta_2O_5	2	THF	50	140	2	No reaction
10	SiO_2	2	THF	50	90	6	No reaction
11	WO_3	2	THF	50	150	8	No reaction
12	GeO_2	2	THF	50	150	8	No reaction
13	ZrO_2	2	THF	50	150	6	No reaction
14*	SnO_2	2	THF	50	150	6	No reaction
15*	Nb_2O_5	2	THF	3	200	4	Starting material + baseline material
16*	ZnO	4	THF	3	200	4	Starting material + baseline material
17*	SiO_2	4	THF	3	200	4	Starting material + baseline material
18*	-	-	THF	3	200	4	Starting material + baseline material

* - reactions were carried out in PARR reactors

Without any success in our attempt to rearrange mono-*O*-methylphloroglucinol to 2,4,6-trihydroxytoluene, alternative approach to the synthesis of 2,4,6-trihydroxytoluene directly from phloroglucinol was investigated.

Direct C-methylation of phloroglucinol with different methylation agents, such as MeI, dimethyl carbonate (DMC), and dimethyl sulfate (DMS), was attempted and it was found that 2,4,6-Trihydroxytoluene could be synthesized effectively in high yields via direct C-methylation of phloroglucinol using MeI as methylating agent. Optimization of the reaction conditions suggested that the reaction is best carried out at 70°C in a $CH_3CN:H_2O$ solvent mixture with MeI as the methylating agent and NaOH as a base to provide about 60% of the desired product with about 33% of the starting material, phloroglucinol, remained unconsumed, Table 4 (entry 18-19).

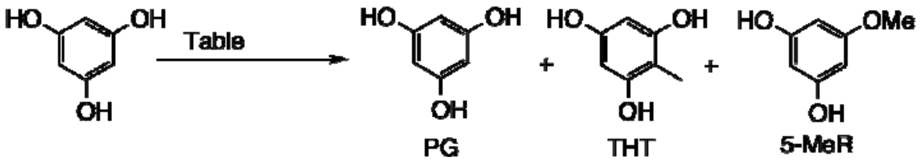


Table 4. C-methylation of phloroglucinol with MeI, dimethyl carbonate (DMC), and dimethyl sulfate (DMS) under various reaction conditions

			Methylating Agents										
	PG	NaOH[a]	MeI	DMC	DMS	Solvents (mL)			T	Time	Results (% yield)[b]		
Entry	(mg)	uL (eq)	uL	uL	uL	H_2O	THF	CH_3CN	°C	(h)	PG	THT	5-MeR
1	100	124 (1)	77	-	-	-	2	-	0	8	43	3	-
2	100	124 (1)	77	-	-	2	-	-	0	8	38	4	
3	100	124 (1)	-	100	-	-	2	-	0	8	46		
4	100	124 (1)	-	100	-	2	-	-	0	8	6		
5	100	124 (1)	77	-	-	-	2	-	rt	8	44	3	0.3
6	100	124 (1)	77	-	-	2	-	-	rt	8	34		
7	100	124 (1)	-	100	-	-	2	-	rt	8	43		
8	100	124 (1)	-	100	-	2	-	-	rt	8	29		
9	500	620 (1)	385	-	-	-	5	-	70	4	18	41	4
10	500	620 (1)	385	-	-	5	-	-	70	4	24	30	0.7
11	500	620 (1)	-	500	-	-	5	-	70	4	45		
12	500	620 (1)	-	500	-	5	-	-	70	4	29		
13	500	1240 (2)	385	-	-	5	-	-	70	2	29	8	
14	500	1240 (2)	385	-	-	5	-	-	70	2	26	14	
15	500	1240 (2)	-	2000	-	-	5	-	70	2	17		
16	500	1240 (2)	-	2000	-	-	-	5	70	2	8		
17	1000	1240 (2)	772	-	-	20	-	-	70	6	21	24	2
18	100	174 (1.4)	116	-	-	5	-	5	70	2	33	60	6
19	100	174 (1.4)	116	-	-	5	-	5	70	0.5	33	58	5
20	108	174 (1.3)	-	-	83	8	-	2	70	1	31	7	27
21	108	174 (1.3)	-	-	83	8	-	2	70	1	32	8	27

[a] = 5 M NaOH; [b] GC yields; PG = Phloroglucinol; THT = 2,4,6-trihydroxytoluene; 5-MeR = 5-methoxyresorcinol; MeI = Methyl iodide; DMC = dimethyl carbonate; DMS = dimethyl sulfate; rt = room temperature

Under the above reaction conditions, only a small amount of byproducts, with about 6 % of 5-methoxyresorcinol formed as detected by GC. The only major drawback of this reaction at the moment is the incomplete conversion of the starting material, phloroglucinol, to 2,4,6-trihydroxytoluene. Consistently, about 30% of phloroglucinol remained unreacted and this amount of unreacted phloroglucinol has been a major problem in purifying 2,4,6-trihydroxytoluene.

Other methylating agents, such as dimethyl sulfate, dimethyl carbonate, and orthomethyl formate, have also been investigated but failed to provide detectable amount of the desired product, 2,4,6-trihydroxytoluene.

Conclusions and Implications for Future Research/Implementation

In route to the microbial synthesis of *O*-methylphloroglucinols, *POMT* from *Rosa chinensis* var. *spontanea* has been successfully de novo synthesized in codon-optimized form for expression in *E. coli*, which is the host currently used for microbial synthesis of PG from glucose. The specific activity of heterologously-expressed, codon-optimized POMT is 5-fold higher than the specific activity of POMT purified to homogeneity from *Rosa chinensis* var. *spontanea* petals. Efforts to create a microbe that converts methoxyresorcinol to dimethoxyphenol had also been made. Protein sequence of orcinol *O*-methyltransferase from *Rosa hybrida* was obtained from GenBank, codon optimized and back-translated into DNA sequence for *E. coli* expression. The resulting OOMT1 gene was synthesized, cloned into the expression vector pET22b and assayed for methylase activity using methoxyresorcinol as substrate. The crude extract specific activity of this construct BL21(DE3)/pKIT1.009 was found to be 0.006 U/mg. The production of dimethoxyphenol in the reaction mixture was further characterized by GC/MS.

Efforts had been made to invent and improve the microbial synthesis of methoxyresorcinol in the fermentor. Draths Corporation had constructed a methoxyresorcinol producing *E. coli* PG1/pKIT1.008 that synthesized 0.7 g/L methoxyresorcinol in the medium under fed-batch fermentor-controlled conditions. It is believed that methoxyresorcinol is toxic to *E. coli* cells and difficulties were experienced in maintaining a healthy culture in the fermentor. To circumvent this issue, resin-based extractive fermentation was carried out as an external loop throughout the run. By using a strong anion-exchange resin under optimized conditions, *E. coli* PG1/pKIT1.008 synthesized 2 g/L methoxyresorcinol in 70 h after inoculation, which is equivalent to a three-fold improvement in titer.

We have successfully demonstrated that the synthesis of mono-, di-, and tri-*O*-methylphloroglucinol was achievable in high yields by reacting phloroglucinol with methanol for 2 h at 140ºC in a sealed-tube in the present of an acid catalyst such as H_2SO_4. The process provided a greater than 90% of O-methylated phloroglucinol with 53% of mono-O-methylphloroglucinol, 35% of di-O-methylphloroglucinol, and 3% of tri-O-methylphloroglucinol. These O-methylated phloroglucinols have been successfully converted to TATB by scientists at ATK Space Systems with yields that are as competitive as what been reported previously (see ATK Space Systems report in the appendix section of this report for more detail) and at the same time reducing large amount of toxic reagents use and minimizing the amount of waste byproducts generated.

The successful conversion of bio-derived phloroglucinol to 2-methylphloroglucinol also provides another attractive "green" approach to the synthesis of TNT without generating any "red water" waste streams associated with the current traditional method of TNT synthesis. Under optimized reaction conditions, the conversion of phloroglucinol to 2-methylphloroglucinol is achievable in a yield of 60% with about 33% of phloroglcinol remained unreacted and a small amount of dialkylated byproduct, 5-methoxyresorcinol, formed, Table 4 entry 18.

With the ease of 2-methylphloroglucinol synthesis from phloroglucinol at hand, the conversion of 2-methylphloroglucinol to TNT was investigated by scientists at ATK Space Systems. It was found that TNT can be easily made by first treating the bio-derived 2-methylphloroglucinol with aqueous hydroxylamine to generate 2,4,6-trioxime-methylcyclohexane followed by refluxing this material in concentrated nitric acid. The process shows that TNT can be made successfully without the use of toxic nitration reagent and eliminates the problematic red water issues. These preliminary results are encouraging signs that phloroglucinol can potential play an important role in the synthesis of high energetic materials such as TNT and TATB without generating much of the toxic wastes associated with the traditional method. Additional research is needed to improve and enhance the product yields and selectivity of this process.

Summary:

1. Draths developed and demonstrated an extractive fermentation for the production of phloroglucinol by the fermentation of glucose.
2. Draths cloned the genes for mono-O-methylation and for di- and tri-O-methylation, and proved they could be functionally expressed.
3. The gene for mono-O-methylation was successfully inserted into the organism producing phloroglucinol and an extractive fermentation was run which demonstrated the production of O-methyl phloroglucinol.
4. The work successfully demonstrated the chemical production of mono-, di-, and tri-O-methylphloroglucinol.
5. Methylation of phloroglucinol yielded 2-methyl phloroglucinol
6. ATK successfully performed nitrations on Draths' O-methyl phloroglucinol derivatives, and showed their utility for the production of TABT and the utility of 2-methyl phloroglucinol for the production of TNT.

Future work to be performed:

1. Complete the construction of an organism containing both the POMT and OOMT genes
2. Develop an efficient fermentation of tri-O-methyl phloroglucinol using the above organism, following the demonstrated extractive techniques.

A. Supporting Data: Contained in the report.

B. List of Scientific/Technical Publications: None. This work was done entirely to produce patentable technology, not for academic publication.

C. Other Supporting Materials: U.S. provisional patent application was filed with the US patent office on July 2, 2010; subject, "Phloroglucinol Synthases and Methods of Making and Using Same;" and inventors Craig Banotai, John Frost, Man K. Lau, Betsy Lehner, and Justas Jancauskas.

Appendices

APPENDIX I.

Table 5a. Optimization of Codon Usage for Expression of the *Rosa chinensis* var. *spontanea* *POMT* in *E. coli*.

		E. coli B frequency	native POMT number	native POMT frequency	optimized POMT number	change
A	GCG	0.36	1	0.05	8	7
	GCC	0.28	7	0.33	6	-1
	GCA	0.21	5	0.24	4	-1
	GCU	0.15	8	0.38	3	-5
H	CAU	0.56	6	0.50	7	1
	CAC	0.44	6	0.50	5	-1
I	AUU	0.48	10	0.43	11	1
	AUC	0.44	7	0.30	10	3
	AUA	0.08	6	0.26	2	-4
K	AAA	0.77	11	0.52	16	5
	AAG	0.23	10	0.48	5	-5
S	AGC	0.25	4	0.14	7	3
	UCG	0.20	1	0.03	6	5
	AGU	0.16	5	0.17	5	0
	UCU	0.15	8	0.28	4	-4
	UCC	0.14	4	0.14	4	0
	UCA	0.10	7	0.24	3	-4

		E. coli B frequency	native POMT number	native POMT frequency	optimized POMT number	change
C	UGC	0.58	4	0.57	4	0
	UGU	0.42	3	0.43	3	0
D	GAU	0.66	14	0.70	13	-1
	GAC	0.34	6	0.30	7	1
L	CUG	0.45	5	0.13	18	13
	UUG	0.15	11	0.28	6	-5
	UUA	0.14	2	0.05	5	3
	CUC	0.12	10	0.26	5	-5
	CUU	0.11	9	0.23	4	-5
	CUA	0.03	2	0.05	1	-1
M	AUG	1.00	11	1.00	11	0
T	ACC	0.47	7	0.41	8	1
	ACG	0.27	0	0.00	5	5
	ACU	0.14	3	0.18	2	-1
	ACA	0.12	7	0.41	2	-5

APPENDIX II.

Table 5b. Optimization of Codon Usage for Expression of the *Rosa chinensis* var. *spontanea* *POMT* in *E. coli*.

		E. coli B frequency	native POMT number	native POMT frequency	optimized POMT number	change
E	GAA	0.62	11	0.46	15	4
	GAG	0.38	13	0.54	9	-4
F	UUU	0.61	9	0.56	10	1
	UUC	0.39	7	0.44	6	-1
N	AAU	0.57	8	0.44	10	2
	AAC	0.43	10	0.56	8	-2
P	CCG	0.61	6	0.30	12	6
	CCA	0.18	6	0.30	4	-2
	CCU	0.14	5	0.25	3	-2
	CCC	0.07	3	0.15	1	-2
Q	CAG	0.65	7	0.64	7	0
	CAA	0.35	4	0.36	4	0
V	GUG	0.43	5	0.19	11	6
	GUU	0.25	11	0.41	7	-4
	GUC	0.18	7	0.26	5	-2
	GUA	0.14	4	0.15	4	0
W	UGG	1.00	5	1.00	5	0

		E. coli B frequency	native POMT number	native POMT frequency	optimized POMT number	change
G	GGC	0.41	6	0.21	11	5
	GGU	0.30	5	0.17	9	4
	GGG	0.19	7	0.24	6	-1
	GGA	0.10	11	0.38	3	-8
R	CGC	0.40	0	0.00	5	5
	CGU	0.35	1	0.08	5	4
	CGG	0.11	3	0.23	1	-2
	CGA	0.05	1	0.08	1	0
	AGA	0.05	1	0.08	1	0
	AGG	0.04	7	0.54	0	-7
Y	UAU	0.69	4	0.50	6	2
	UAC	0.31	4	0.50	2	-2
stop	UAA	0.64	0	0.00	1	1
	UGA	0.27	1	1.00	0	-1
	UAG	0.09	0	0.00	0	0

APPENDIX III. ATK Launch System Final Report

Manufacture of TATB and TNT from Biosynthesized Phloroglucinols

SERDP Project Number WP-1582

Final Report

Prepared For:
SERDP Program Office
&
Draths Corporation

Prepared by:
Nicholas A. Straessler, PhD
Scientist, ATK Space Systems
October 13, 2009

ABSTRACT

Alternative syntheses for the common explosives 1,3,5-triamino-2,4,6-trinitrobenze (TATB) and 2,4,6-trinitrotoluene (TNT) were developed in an effort to minimize the environmental impact of their production. Bioderived mono-, di- and tri-*O*-methylphloroglucinol (5-methoxyresorcinol, 3,5-dimethoxyphenol, and 1,3,5-trimethoxybenzene respectively) were individually converted to TATB in moderate to good yields demonstrating potential new pathways that are competitive with the known synthetic routes. The processes involve nitration of the aromatics to their trinitro analogs followed by *O*-methylation (where applicable) to afford 1,3,5-trimethoxy-2,4,6-trinitrobenzene that upon treatment with ammonia gives TATB. The new starting materials are substitutes for the historical TATB precursors 1,3,5-tirchlorobenzene and phloroglucinol, and they enable between 33-100% reductions in organic alkylating agent compared to the synthesis of TATB from phloroglucinol. Studies investigating TATB particle formation and morphology were also conducted. Results indicate that the length of the alkyl ether chain and reaction conditions play important roles in the amination reactions leading to TATB.

TNT was synthesized by non-nitrative techniques in an attempt to eliminate red water waste streams that accompany its conventional production. Bioderived 2-methylphloroglucinol was treated with aqueous hydroxylamine to afford 2,4,6-trioxime-methylcyclohexane. Oxidation of this material with refluxing nitric acid yielded moderate amounts of crude TNT. The product was difficult to isolate in the solid state, but NMR spectroscopy results confirm its presence. Despite the isolation issues, this route demonstrates potential new strategies for TNT production that could eventually eliminate the current environmental waste problems.

Definition of terms and abbreviations used interchangeably in this report:
Phloroglucinol (PG) = 1,3,5-trihydroxybenzene
2-Methylphloroglucinol = 2,4,6-trihydroxytoluene
2-Methylphloroglucinol trisoxime = 2,4,6-trisoxime methylcyclohexane
Trinitrophloroglucinol (TNPG, **1**) = 1,3,5-trihydroxy-2,4,6-trinitrobenzene
Mono-*O*-methylphloroglucinol = 5-methoxyresorcinol (MR)
Di-*O*-methylphloroglucinol = 3,5-dimethoxyphenol (DMP)
Tri-*O*-methylphloroglucinol = 1,3,5-trimethoxybenzene (TMB)
2,4,6-Trinitro-mono-*O*-methylphloroglucinol = 5-methoxystyphnic acid (MSA, **2**)
2,4,6-Trinitro di-*O*-methylphloroglucinol = 3,5-dimethoxypicric acid (DMPA, **3**)
Tri-*O*-methylphloroglucinol = 1,3,5-trimethoxy-2,4,6-trinitrobenzene (TMTNB, **4**)
1,3,5-Triethoxy-2,4,6-trinitrobenzene = TETNB
3,5-Dimethoxy-2,4,6-trinitro-propoxybenzene = DMTNPB
See Appendix I for graphical representations of selected compounds.

OBJECTIVES

The overall objective of this research was to demonstrate new synthetic routes to two existing explosives, TATB and TNT. The purpose of developing new synthetic routes was to reduce the environmental concerns associated with production of both of these materials. The specific objectives of this contract that were assigned to ATK are described below followed by brief descriptions of their relevance to TATB and TNT syntheses.

1. Chemically nitrate bioderived mono-, di-, and tri-*O*-methylphloroglucinol.
2. Chemically convert 2,4,6-trinitro-mono-*O*-methyl- and 2,4,6-trinitro-di-*O*-methylphloroglucinol by reaction with trimethyl orthoformate and then ammonia to TATB.
3. Chemically convert bioderived 2-methylphloroglucinol to its trisoxime.
4. Oxidize 2-methylphloroglucinol trisoxime to TNT.

TATB

The goal of this research was to replace 1,3,5-trichlorobenzene and phloroglucinol with phloroglucinol methyl ethers produced from renewable resources as starting materials for TATB. This would in turn provide environmentally friendly synthetic alternatives to existing routes to TATB. The new syntheses were proposed based on modification of a known route to TATB starting with phloroglucinol.[1] In addition to eliminating the hazards associated with toxic 1,3,5-trichlorobenzene, bioderived *O*-methylphloroglucinol starting materials would aid in: 1) reducing the dependence on petroleum derived chemical reagents, 2) reducing the total mass/volume of the waste streams by increasing atom efficiency, and 3) making progress toward better understanding TATB particle formation so as to identify a new synthetic process that can be qualified to meet the military specifications of TATB.

TNT

The primary objective of this research was to eliminate red water waste streams commonly linked to TNT production methods. In order to accomplish this task, a new synthesis of TNT was envisioned following established chemistry used to convert phloroglucinol to trinitrobenzene by non-nitrative methods.[2] It was hypothesized that analogous reactions could be performed to convert bioderived 2-methylphloroglucinol to TNT. The outcome would ultimately be a red water free synthesis of TNT.

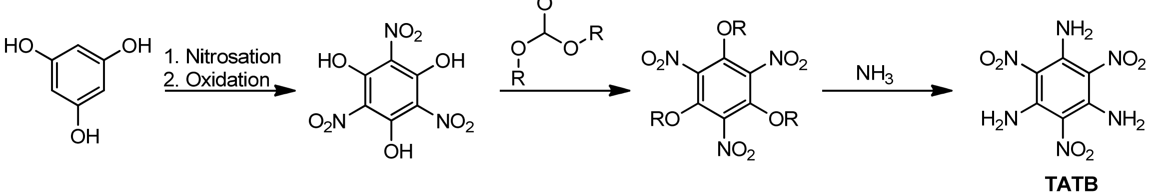
BACKGROUND

TATB

TATB is a unique explosive used in insensitive munitions.[3] It is a high density material with unparalleled resistance to heat and external stimuli. As a result, it is used in military applications where extreme safety is absolutely critical. TATB was once supplied to the United States Department of Defense (DoD) by Royal Ordnance (UK), but a recent policy change now prohibits that manufacturer from providing TATB to any entity besides the UK Atomic Weapons Establishment. Consequently, the United States now has no qualified supplier of TATB and as a result, future sources of the material are in question.

TATB derived from 1,3,5-trichlorobenzene (Benziger process)[4] is the only material that has been qualified and proven to be satisfactory for all DoD applications.[5] However, the environmental hazards of 1,3,5-trichlorobenzene and its associated waste streams have spawned numerous attempts to synthesize TATB from other starting materials. These efforts have culminated in the manufacture of high-quality TATB on the production scale, but subsequent performance testing has not been satisfactory in all configurations. The processing and performance of TATB is dependent on chemical purity, as well as particle size, and morphology.

Bellamy *et al*[1] devised an alternative synthesis of TATB (Bellamy process) that circumvents the use of halogenated aromatics by starting with phloroglucinol, Scheme 1. Draths Corporation further reduced the environmental impact of this synthesis by demonstrating the ability to produce phloroglucinol biosynthetically from glucose.



Scheme 1. Synthesis of TATB from phloroglucinol by the Bellamy process, R = Me, Et, or Pr.

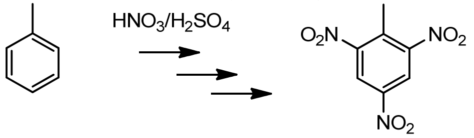
Although the Bellamy process reflects clear advantages over the Benziger process, from an environmental standpoint it still suffers from substantial dependence on petroleum derived organic alkylating agents, specifically alkyl orthoformates ($HC(OR)_3$), for etherification following nitration. In their report, Bellamy *et al*, perform the etherification as a solvolysis type reaction where the orthoformate is both the reactant and the solvent.[1] Previous research at ATK in 2002 as part of a Manufacturing Technology (ManTech) contract from the DoD improved upon the original Bellamy process by reducing trialkyl orthoformate usage to only 2 mol-equivalents per hydroxyl group.[6] This translates to six mol-equivalents of trialkyl orthoformate for conversion of phloroglucinol to TATB.

As part of the current work, attention was directed toward improving the Bellamy process even more by investigating the synthesis of TATB from *O*-methylphloroglucinol derivatives, namely 5-methoxyresorcinol (MR), 3,5-dimethoxyphenol (DMP), and 1,3,5-trimethoxybenzene (TMB). The impetus for using these compounds as starting materials in the synthesis of TATB is twofold. First, like phloroglucinol, they have all been biosynthetically derived from renewable resources at Draths Corporation; second, they were expected to reduce the demand for alkylating agent in direct proportion to their respective degrees of *O*-methylation. This in turn would correspond to a 33%, 66%, and 100% savings on alkylating agent for MR, DMP, and TMB respectively compared to the synthesis of TATB from phloroglucinol

Research on the ManTech contract at ATK demonstrated that nitration of phloroglucinol at the production scale could not be accomplished practically with mixed acid.[6] However, it was discovered that a method of nitrosation/oxidation described in the patent literature[7] was scalable. Therefore, the production-scale process for the synthesis of TATB currently used at ATK relies on the preliminary deprotonation with sodium hydroxide of at least one of the hydroxyl groups of phloroglucinol to render it soluble in aqueous solution. The resulting sodium phloroglucinate is then subjected to nitrosation with sodium nitrite and nitric acid followed by N-oxidation to generate trinitrophloroglucinol. After nitration, this compound is *O*-ethylated with triethyl orthoformate in toluene. Finally TATB is generated by amination with ammonia gas and concomitant displacement of ethanol.

TNT

TNT is a common energetic compound used extensively as the melt phase in a variety of melt-pour explosive formulations.[3] Its melting point (80 °C) is much lower than its spontaneous detonation temperature which makes it ideal for such applications. It is particularly attractive for long term military use because it is highly hydrophobic and relatively insensitive to friction and shock (although not as insensitive as TATB). TNT is a legacy Army explosive and is often used as an industry standard by which other explosives are compared in terms of energetic performance and stability. The synthesis of TNT involves the stepwise nitration of toluene with a mixture of nitric acid (HNO_3) and sulfuric acid (H_2SO_4), Scheme 2.



Scheme 2. Conventional synthesis of TNT.

During TNT recovery and purification, sellite (sodium sulfite) is typically added to the process stream to remove off-isomers of TNT. The resulting waste stream of sulfonated byproducts is referred to as ‘red water’. Despite significant efforts to remediate red water and reduce its production, it remains a serious environmental problem and is still a concern to the US Army.

An alternative synthesis of TNT starting with bioderived 2-methylphloroglucinol (2,4,6-trihydroxytoluene) was proposed to eliminate red water waste streams. The reaction is based on a recently published conversion of phloroglucinol to trinitrobenzene (TNB) via a stable trisoxime intermediate.[2] The process is an intriguing method for generating nitroaromatics that does not involve nitration reactions. It takes advantage of the keto-enol tautomerization of phenols which provides an electrophilic carbonyl on the ring that is available for attack by hydroxylamine. In the case of phloroglucinol, the resulting trisoxime cyclohexane was oxidized with nitric acid to TNB. The parallel chemistry with 2-methylphloroglucinol was investigated in the context of this work.

MATERIALS and METHODS

General

All reagents were purchased from commercial chemical suppliers (VWR International and Aldrich Chemical Company) and used as received. 5-Methoxyresorcinol, 3,5-dimethyoxyphenol, 1,3,5-trinitrobenzene were supplied by Draths Corporation, and additional quantities were purchased commercially. The NMR solvents CD_2Cl_2, $CDCl_3$, d_6-DMSO, and acetone-d_6 were obtained from Cambridge Isotopes Laboratories. Water was purified in-house using a typical double distillation apparatus. ^{1}H and ^{13}C NMR data were collected on a JEOL Eclipse+ 400 MHz spectrometer; the chemical shifts are reported in δ (ppm) relative to residual solvent peaks ($CDCl_3$ δ ^{1}H 7.25, ^{13}C 77.00; CD_2Cl_2 δ ^{1}H 5.31; ^{13}C 53.08; d_6-DMSO δ ^{1}H 2.50; acetone-d_6 δ ^{1}H 2.05). Melting points are uncorrected and were determined using the standard open capillary method. Elemental analyses were performed on a Perkin Elmer Model 2400 Series II CHNS/O analyzer. FT-IR spectra were collected on a Thermo Scientific Nicolet 6700 FT-IR spectrometer.

Synthesis and Characterization

2,4,6-trinitrophloroglucinol monohydrate (1). A round bottom flask was charged with 96% sulfuric acid (30 mL) and chilled to approximately 5 ^oC. Ammonium nitrate (1.48 g, 18.48 mmol) was added with stirring at a rate such that the temperature did not rise above 10 ^oC. Once the solution temperature returned to 5 ^oC, phloroglucinol dihydrate (PGDH, 1.00 g, 6.16 mmol) was added at a rate such that the temperature did not increase beyond 10 ^oC. Vigorous stirring was maintained to visibly prevent concentrating the solid in the center vortex. After PGDH addition was complete and the associated exotherm began to subside, the cooling bath was removed and the reaction was stirred for an additional 10 min. The heterogeneous yellow mixture was added in one portion to crushed ice (100 g) and was stirred gently until all the ice had melted. The resulting precipitate was isolated by vacuum filtration, washed with cold 10% HCl, and air dried to afford 2,4,6-trinitrophloroglucinol monohydrate (1.57 g, 91%), as a yellow solid. Melting point, ^{1}H and ^{13}C NMR spectra matched those previously reported.[8]

5-methoxystyphnic acid (2). Similar to (**1**) above, ammonium nitrate (1.72 g, 21.48 mmol) and 5-methoxyresorcinol (1.00 g, 7.12 mmol) were used in the reaction. The resulting precipitate was isolated by vacuum filtration, washed with cold 10% HCl, and air dried to afford 5-methoxystyphnic acid (1.89 g, 96%) as a light yellow solid, mp = 165 – 167 ^oC (decomp.): ^{1}H NMR (CD_2Cl_2) δ 4.09 (s, 3H, CH_3), 11.37 (s, 2H, OH); ^{13}C NMR (CD_2Cl_2) δ 61.99, 118.34, 126.20, 151.22, 152.02. Elemental analysis calculated for $C_7H_5N_3O_9$: C, 30.56; H, 1.83; N, 15.27. Found: C, 30.12; H, 1.54; N, 14.83. Slow diffusion of *n*-heptane into a chloroform solution of **2** afforded high purity X-ray quality single crystals of **2** (Appendix III).

3,5-dimethoxypicric acid (3). A round bottom flask was charged with 96% sulfuric acid (10 mL) and chilled to approximately 5 ^oC. Sodium nitrate (410 mg, 4.82 mmol) was added with stirring at a rate such that the temperature did not rise above 10 ^oC. Once the solution temperature returned to 5 °C, 3,5-dimethoxyphenol (250 mg, 1.62 mmol) was added at a rate such that the temperature did not increase beyond 10 °C. Vigorous stirring was maintained to visibly prevent concentrating the solid in the center vortex. After DMP addition was complete and the associated exotherm began to subside, the cooling bath was removed and the reaction was stirred for an additional 20 min. The dark red solution was added in one portion to a mixture of ice (75 g) and water (75 g) and was stirred gently until all the ice had melted. The resulting precipitate was isolated by vacuum filtration, washed with cold 10% HCl, and air dried to yield 3,5-dimethoxypicric acid (400 mg, 85%) as a light yellow solid, mp = 75-76 °C: ^{1}H NMR ($CDCl_3$) δ 4.05 (s, 6H,CH_3), 11.25 (br s, OH); ^{13}C NMR ($CDCl_3$) δ 63.83, 127.53, 133.47, 150.57, 150.79. Elemental analysis calculated for $C_8H_7N_3O_9$: C, 33.23; H, 2.44; N, 14.53. Found: C, 33.25; H, 2.17; N, 14.37. Slow evaporation of an *n*-heptane solution of **3** yielded high purity X-ray quality single crystals of **3** (Appendix III).

1,3,5-trimethoxy-2,4,6-trinitrobenzene (4). A round bottom flask was charged with 96% sulfuric acid (10 mL) and chilled to approximately 5 °C in an ice bath. Potassium nitrate (450 mg, 4.45 mmol) was added with stirring at a rate such that the temperature did not rise above 10 °C. After addition was complete and the temperature returned to 5 °C, 1,3,5-trimethoxybenzene (250 mg, 1.49 mmol) was added at a rate such that the temperature did not increase beyond 10 °C. Vigorous stirring was maintained to visibly prevent concentrating the solid in the center vortex. After TMB addition was complete and the associated exotherm began to subside, the reaction was warmed to 50 °C (5 °C/min). The dark red solution was added in one portion to crushed ice (60 g). The mixture was stirred until all the ice had melted and the resulting precipitate was isolated by vacuum filtration, washed with distilled water, and air dried to yield 1,3,5-trimethoxy-2,4,6-trinitrobenzene (270 mg, 62%) as a light yellow solid. Melting point, ^{1}H and ^{13}C NMR spectra matched those previously reported.[9] Slow evaporation of an *n*-heptane solution of **4** yielded high purity X-ray quality single crystals of **4** (Appendix III).

Alternatively, **4** was recovered by treating **2** and **3** with 4 and 2 mol-equivalents respectively of trimethyl orthoformate in just enough toluene to dissolve the solids. The mixtures were heated to reflux and monitored by TLC (ethyl acetate) until the reactions were complete. Reactions typically took longer than 8 h, but heating could be stopped and restarted without noticeable negative effects on the product. After reactions were complete by TLC, solutions were brought to dryness by evaporation. The resulting yellow solids were washed with 1% NaOH, filtered and dried to yield pure **4** (80-95% yields) as yellow solid. Melting points, ^{1}H and ^{13}C NMR spectra matched those previously reported.[9]

3,5-dimethoxy-2,4,6-trinitro-propoxybenzene. 4 (2 g, 6.9 mmol) and tripropyl orthoformate (3 mL, 13.85 mmol) were combined in a round bottom flask and heated with stirring to 105 °C for 3 h. The resulting clear yellow solution was added to a plug of dry silica gel which was then washed with hexanes (150 mL). The yellow product still visible on the silica gel was removed with ethyl acetate (200 mL). Slow evaporation of the ethyl acetate solution in a fume hood afforded 3,5-dimethoxy-2,4,6-trinitro-propoxybenzene (2.1 g, 91% yield) as a slow crystallizing yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H, propyl CH_3), 1.73 (m, 3H, propyl CH_2), 4.02 (s, 6H, O-CH_3), 4.10 (t, 2H, propyl CH_2); ^{13}C NMR ($CDCl_3$) δ 9.78, 23.14, 64.48, 79.68, 135.55, 135,99, 146.68, 147.19. Elemental analysis calculated for $C_{11}H_{13}N_3O_9$: C, 39.89; H, 3.96; N, 12.69. Found: C, 40.06; H, 3.63; N, 12.41.

1,3,5-triamino-2,4,6-trinitrobenzene. TATB was synthesized by the following general method. 1,3,5-Trialkoxy-2,4,6-trinitrobenzene (0.5 g, alkoxy = methyl, ethyl, dimethyl-propyl) was dissolved in toluene (5 mL). The solution was heated in a Parr reactor to 90 ^{o}C with stirring. At 90 ^{o}C the vessel was pressurized to 60 psi of ammonia following a 2 min purge. After 3 h at these conditions heating was stopped and the vessel was depressurized. The resulting precipitate was isolated by vacuum filtration and rinsed with toluene to afford TATB characterized by FT-IR (see Appendix II) and HPLC (see Results and Discussion section). Yields ranged from 75-85% TATB regardless of the starting material. Yields of TATB greater than 90% were achieved when the reaction was carried out with at least 1 g of starting material.

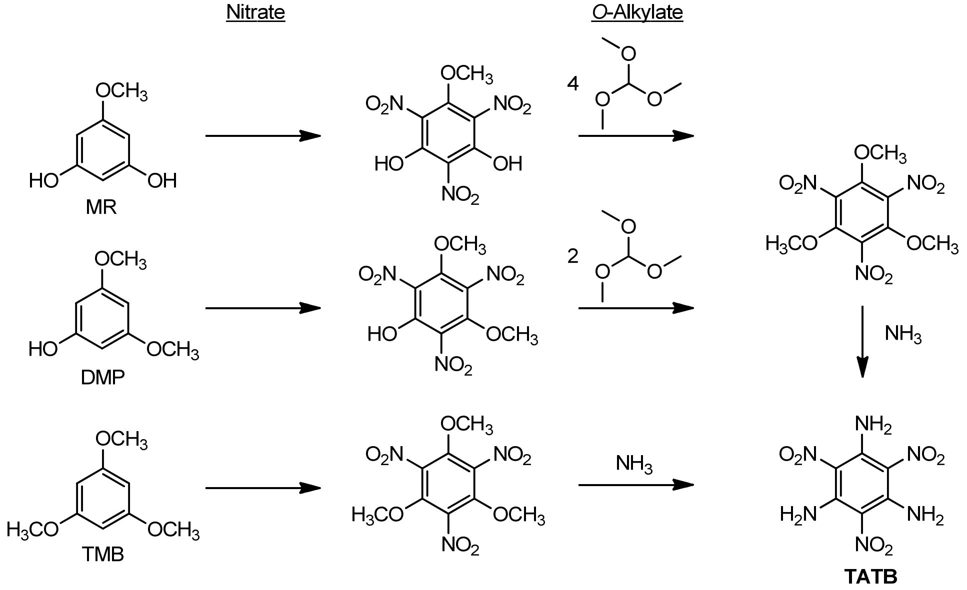
1-methyl-2,4,6-trioximecyclohexane. Methylphloroglucinol (1.0092 g; 7.7 mmol) was placed in a 10 mL one-neck round-bottom flask equipped with a Teflon stir bar. A 50% by weight aqueous solution of hydroxylamine (4.2601 g; 64 mmol) was added to the flask. Methylphloroglucinol completely dissolved after 5 min of stirring. The stir bar was removed, and the solution was let stand at ambient conditions. After 4 hr a light tan product had precipitated. The solids were filtered, washed with water, and air dried (yield 0.9093 g; 64%). The ^{1}H NMR spectrum of the product in d_6-DMSO showed three peaks of approximately equal intensity at 10.81, 10.83, and 10.84 ppm. These peaks were not present when D_2O was added to the NMR tube, indicating that they can be assigned to the hydroxyl protons. The spectrum also contains approximately seven peaks between 3.20 and 3.47 ppm corresponding to the aliphatic protons and two peaks of approximately equal intensity at 1.16 and 1.17 ppm corresponding to the methyl protons.

2,4,6-Trinitrotoluene. 2,4,6-Trioxime-methylcyclohexane (0.5017 g, 2.7 mmol) was ground in a mortar and pestle with CH_2Cl_2 (15 mL) to form a suspension. Concentrated HNO_3 (24 g, 90%) was chilled in an ice bath in a 3-necked round-bottom flask under N_2. The suspension was added dropwise with stirring to the cooled HNO_3. A small amount of nitrogen oxide fumes was produced upon each addition, which was swept away by N_2. The fuming was allowed to subside before adding the next aliquot. After all of the trioxime had been added, the flask was fitted with a reflux condenser and transferred to a bath held at 55 ºC. Copious nitrogen oxide fumes were generated upon reflux and were swept away by N_2. At the end of 3 h, the reaction was quenched with ice, diluted to 200 mL with water, and extracted with ethyl acetate (100 mL). The ethyl acetate extract was washed sequentially with water, $NaHCO_3$ solution, and brine, then dried over $MgSO_4$. The solvent was removed by rotary evaporation, and the resulting brown oil was flash chromatographed with 50:50 $CHCl_3$:acetone through silica. A brown oil was obtained upon removing the solvent, and a brown fraction remained on the silica. The ^{1}H NMR spectrum of the brown oil in acetone-d_6 had predominant peaks at 9.00 and 2.67 ppm. A sample of authentic TNT gave identical shifts in acetone-d_6. The ^{1}H NMR spectrum of the reaction product had numerous small impurity peaks. None of the peaks corresponded to the trioxime, and the impurities were not identified.

RESULTS and DISCUSSION

Nitration of Phloroglucinol and its Mono-, Di-, and Trimethyl Ethers

The potential to synthesize TATB from the proposed phloroglucinol methyl ethers, Scheme 3, was critically dependent upon the ability to trinitrate them first.

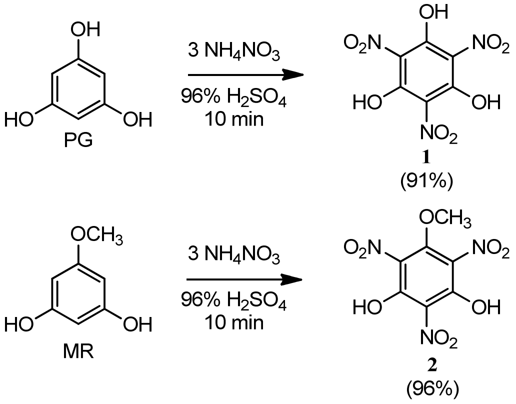


Scheme 3. Synthesis of TATB from *O*-methylphloroglucinol derivatives: MR, DMP, and TMB.

It was not immediately obvious that adding three nitro groups to these starting materials would be possible. The primary concern was the fact that alkoxy substituents are not as activating in electrophilic aromatic substitutions (i.e. nitrations) as hydroxyl groups are. Accordingly, it would hold true that the nitration of the proposed new starting materials would get progressively more difficult as the number of methoxy groups vs. hydroxyl groups increases. Moreover, complete replacement of hydroxyl groups with methoxy substituents, as in 1,3,5-trimethoxybenzene, precludes the option of solublizing the material via preliminary deprotonation. Therefore, various nitration strategies were explored in an attempt to identify one that could be used to successfully tri-nitrate all three of the new phloroglucinol methyl ether starting materials.

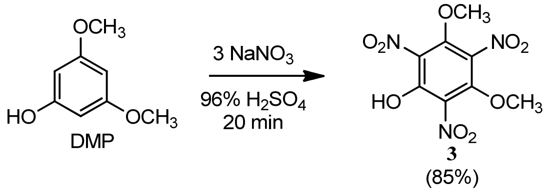
In general, tri-nitration of aromatics is relatively difficult because of the deactivating effect of the first two nitro groups. Di-nitration of aromatic compounds is rather facile, but the harsh conditions required to introduce a third nitro group to the ring can compromise yields as a result of oxidative side reactions.[10] The nitration of phloroglucinol at ATK by nitrosation/oxidation has historically given good yields of trinitrophloroglucinol, so it was the first technique explored to nitrate the *O*-methylphloroglucinol derivatives. However, this method proved insufficient due to excessive foaming. After much experimentation, a variation of the common mixed acid nitrating agent provided the most promising results. Mixtures of a nitrate salt dissolved in sulfuric acid were used to synthesize 5-methoxystyphinic acid (**2**), 3,5-dimethoxypicric acid (**3**), and 1,3,5-trimethoxy-2,4,6-trinitrobenzene (**4**). It was demonstrated that this method could be extended to synthesize trinitrophloroglucinol (**1**) as well. All of the aromatic starting materials were also successfully converted to their trinitro counterparts in yields comparable to those reported here with mixed acids. But the reactions were milder and more controllable using a nitrate salt in place of nitric acid.

Three nitrate salts (XNO_3, where X = NH_4^+, Na^+, K^+) individually dissolved in sulfuric acid (96%) were evaluated as nitrating agents for the four aromatic starting materials. The identity of the nitrate salt had little observed effect on chemical yield and purity of **1** and **2**. PG and MR were both converted to their trinitro counterparts in comparable yields and purities with all three nitrate salts in 10 minutes. Ultimately, ammonium nitrate was the preferred salt due to its rapid dissolution in sulfuric acid and consistently high yields of **1** (91% yield) and **2** (96% yield), Scheme 4.



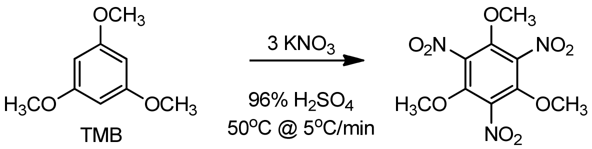
Scheme 4. Synthesis of **1** and **2**.

The identity of starting nitrate salt had a more pronounced effect on the nitrations of DMP and TMB. DMP was most favorably converted to **3** (85% yield) in 20 minutes using sodium nitrate, Scheme 5.



Scheme 5. Synthesis of **3**.

The nitration of 1,3,5-trimethoxybenzene with dinitrogen pentoxide to produce 1,3,5-trimethoxy-2,4,6-trinitrobenzene in either dichloromethane or acetonitrile has been reported in the literature in 65% crude yield. (1) In an effort to keep this research in line with the overall SERDP objectives, the nitration of 1,3,5-trimethoxybenzene was explored using more environmentally friendly conditions, i.e. without organic solvents or the need to dehydrate nitric acid. The best conditions for the synthesis of **4** (62% yield) from TMB included three mol-equivalents of potassium nitrate with heating to 50 °C, Scheme 6. Three mol-equivalents of ammonium nitrate at 45 °C also produced good quality **4**, but yields were slightly better with potassium nitrate. Four mol-equivalents of sodium nitrate were necessary to obtain similar results at 40 °C for 30 minutes. Heating to 65 °C with three mol-equivalents of sodium nitrate gave a 52% yield of **4**.



4
(62%)

Scheme 6. Synthesis of **4**.

Methylation of Trinitro-*O*-methylphloroglucinols With Trimethyl Orthoformate and Subsequent Amination with Ammonia

Theoretically, any trialkyl orthoformate could be used for etherification of compounds **1** - **3**, but the scope of this research was limited to trimethyl-, triethyl-, and tripropyl orthoformates due to prior experience and literature examples[1] of each. The specific effects of alkoxy chain length are addressed in the Particle Size and Morphology section of this report.

TMB would be the optimal precursor to TATB because it does not need to be *O*-alkylated prior to condensation with ammonia to produce TATB. However, yields of **4** from the nitration reactions were substantially lower than **2** and **3**. Ideally, the lower yields resulting from direct nitration of TMB would be offset by not having to alkylate it. Nonetheless, the *O*-methylations of **2** and **3** with trimethyl orthoformate to form **4** were carried out. *O*-methylation in toluene of both **2** and **3** with four and two mol-equivalents of trimethyl orthoformate respectively afforded **4** in good yields (80-95%). These conditions are stoichiometrically analogous to the current ATK TATB production scale synthesis, but proportionally require less alkylating agent. The methylation reactions in toluene are lengthy with respect to the neat solutions reported by Bellamy *et al.*[1] It is important to the progress of the reaction to remove orthoformate byproducts from the solution by fractional distillation during the alkylations. At the scale of this work it was easier to simply heat the reaction flask without a reflux condenser to allow unwanted vapors to escape. The reaction volume was maintained at its original level by repeated addition of solvent as necessary. Under-methylated material was easily removed by washing the water insoluble product, **4**, with dilute (1%) NaOH.

The methylation of **3** with trimethyl orthoformate was also investigated in several other organic solvents including tetrahydrofuran (THF), methanol, methyl-*t*-butyl ether (MTBE), and ethyl acetate. Only the reaction in ethyl acetate gave significant conversion to **4** in a reasonable timeframe (1-3 days). Because the reaction had to be run at a lower temperature in ethyl acetate (due to its lower boiling point) than in toluene, methylations were considerably longer, but were still nearly quantitative. It was determined that the ensuing amination reaction could also be carried out in the ethyl acetate, thereby eliminating the need for intermediate isolation of **4**. It was found that the isolation of **3** could also be eliminated by extracting it too with ethyl acetate from the aqueous precipitation solution following nitration of DMP. Together these steps demonstrate a process for synthesizing TATB from DMP that does not require isolation of any potentially hazardous trinitroaromatics. Unoptimized reactions following this sequence of steps yielded TATB in 72% yield based on DMP, but only 93% purity. Under-aminated species were the main impurity based on HPLC analysis. The amination reactions were carried out under only ~1 psi of ammonia at room temperature. Higher temperatures and pressures would likely improve the purity of TATB derived from **4** in ethyl acetate and is an area of future research interest.

Solid samples of **4** were dissolved in toluene and aminated at 90 °C under 60 psi of ammonia for 3 h. Yields of TATB were typically around 80-85% and increased with scale. The purity of TATB derived from **4** was at best 93%, the major contaminants being the well known impurity ammonium diaminopicrate[11,1] as well as under-aminated product. The amination conditions used had been previously optimized for 1,3,5-triethoxy-2,4,6-trinitrobenzene (TETNB), and no significant effort was devoted towards optimizing the conditions for **4**. Nonetheless, these results are proof that TATB can be synthesized from all three bioderived phloroglucinol methyl ethers.

Methylation of PG Using Dimethyl Carbonate in Ionic Liquids

During the course of this research, the use of dimethyl carbonate (DMC) was explored as a potential *O*-methylating agent. Based on literature results for the alkylation of mono- and dihydroxybenzenes using ionic liquids and DMC,[12] it seemed to follow that this might be an effective and environmentally sound approach for converting phloroglucinol to 1,3,5-trimethoxybenzenes. Phloroglucinol-dihydrate was refluxed with toluene and, by use of a Dean-Stark distillation head, the water was effectively removed. The toluene was removed under reduced pressure to afford anhydrous phloroglucinol. The ionic liquid, 1-*n*-butyl-3-methylimidazolium chloride ([BMIm]Cl), was purchased at two purity levels (95% and 98%) from Aldrich Chemical Company and used as received. High purity DMC was also purchased for the experiments and used as received. Glassware was oven dried and all operations were carried out under a nitrogen atmosphere. In a typical reaction, the mixture was heated in an oil bath at selected temperatures ($\geq$100 ^{o}C). All mixtures turned into a two-liquid phase system where the anhydrous phloroglucinol dissolved in the DMC and the ionic liquid melted (bottom layer). Sampling both layers at several hours and even days did not indicate that alkylation of the phloroglucinol was occurring (no methoxy peaks were observed in the proton NMR spectra). Consequently, it would appear that use of [BMIm]Cl and DMC is not a suitable combination for alkylating phloroglucinol. Methylations of **3** with DMC were also attempted in methanol, in MTBE, neat, and under high temperature and high pressure conditions, all to no avail.

TATB Particle Size and Morphology Studies

Benziger, or Legacy, TATB is a porous crystalline material, Figure 1, with ~70 micron particle size[5] and a 375 ^{o}C DSC onset temperature.[1]

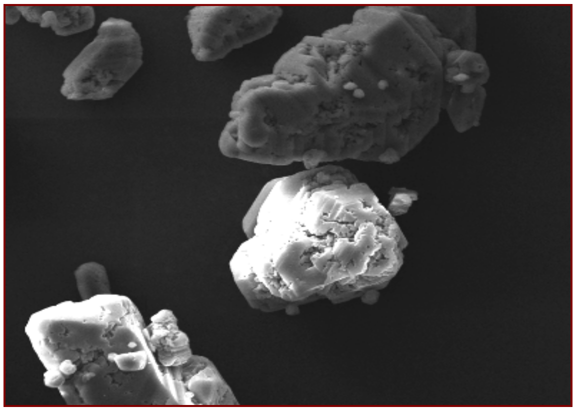


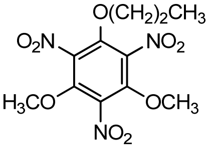
Figure 1. Legacy TATB, courtesy of *NAV-AIR*.

The reasons are not clear why TATB from alternate synthetic routes does not perform the same in all tests as the Legacy TATB. However, there are a few obvious physical differences between the materials including particle size and morphology that have been speculated as the cause of the performance disparity (see Figure 2). Although TATB synthesized at ATK in 2002 on the ManTech contract met the military specifications, the US Navy is reluctant to use it due to its lack of performance in certain tests. A detailed comparison of Legacy and ATK ManTech TATB has been made elsewhere.[5]

Figure 2. ATK ManTech TATB, 2002.

There is a correlation between TATB purity and DSC onset temperature and another correlation has been made between TATB purity and length of the alkoxy chain on the precursor compound.[1] It has been shown that higher purity TATB, with higher DSC onset temperatures, is recovered from trialkoxy-trinitrobenzenes with longer alkyl chains, e.g. propoxy, under certain amination conditions.[1] Unfortunately, synthesizing 1,3,5-tripropoxy-2,4,6-trinitrobenzene (TPTNB) is cost prohibitive at the production scale due to the price of tripropyl orthoformate (TPOF).

To probe the effects of aminating a mixed ether trinitrobenzene derivative, 3,5-dimethoxy-2,4,6-trinitro-propoxybenzene (DMTNPB) was synthesized by treating **3** with tripropyl orthoformate. Specifically, it was of interest to know if DMTNPB yielded TATB more similar in particle size and purity to that derived from **4** or TPTNB. Amination reactions in toluene of **4**, DMTNPB, and TETNB were carried out in parallel at 90 ^{o}C under 60 psi of ammonia for 3 h. All three starting materials produced similar yields of yellow product characterized as TATB by FT-IR (Appendix II). The products were further analyzed by HPLC and DSC. The data conclusively show that DMTNPB behaves more similarly to **4** than it does TPTNB in the synthesis of TATB, Table 1. This information suggests that under these conditions, TETNB is still the most practical candidate for production scale synthesis of TATB.



DMTNPB

Table 1. Analytical data of TATB derived from 3 different starting materials.

Starting Material	Ave % TATB by HPLC	Ave DSC Onset (^{o}C)	Ave 50th Percentile Particle Size (micron)
4	86	370	19
DMTNPB	92	372	25
TETNB	99	377	29

During the course of this research, it was discovered that there was a significant impact on TATB particle size when the aminations were performed without stirring. In general particle sizes nearly doubled with no variation in purity and a slight increase in DSC onset temperature was observed, Table 2.

Table 2. Analytical data of TATB derived from 3 different starting materials without stirring during amination.

Starting Material	Ave DSC Onset (°C)	Ave 50th Percentile Particle Size (micron)
4	370	53
DMTNPB	373	56
TETNB	382	63
Legacy TATB	375[1]	70[5]

Scanning electron microscopy (SEM) images show that TATB synthesized from the three different starting materials are crystalline, but vary in topography, Figure 3.

The newly acquired chemical and physical data combined with the SEM images undoubtedly demonstrate that this research has lead to new synthetic methodologies capable of producing TATB that is comparable, at least in purity, appearance, particle size, and DSC onset temperature, to the Legacy material. Optimally, further manipulation of the amination conditions could lead to successful synthesis of Legacy quality TATB from **4**.

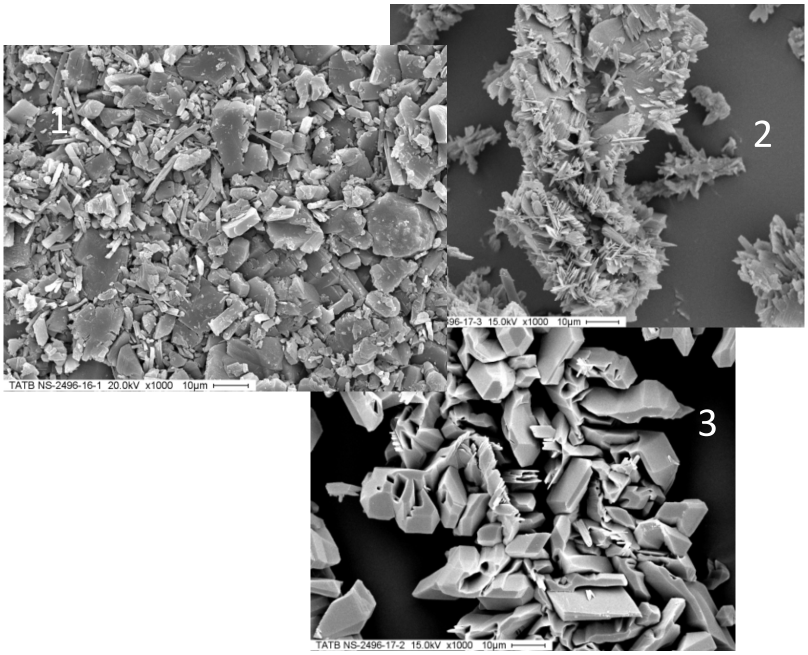
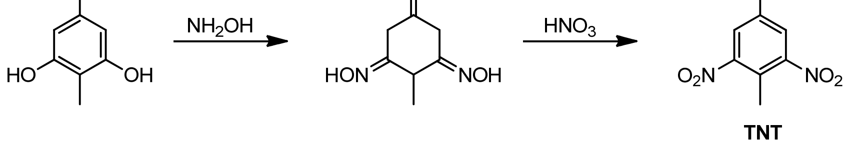


Figure 3. SEM images of TATB derived from 1) **4**, 2) DMTNPB, and 3) TETNB.

TNT

A new method of synthesizing TNT from 2-methylphloroglucinol by non-nitrative techniques was developed, Scheme 7.



Scheme 7. Synthesis of TNT from methylphloroglucinol

The synthesis of 2,4,6-trioxime-methylcyclohexane from 2-methylphloroglucinol was straightforward and precipitation of the product made for easy recovery. The yields of this step (64%) were somewhat disappointing, but could likely be increased with scale. The oxidation of 2,4,6-trioxime-methylcyclohexane to TNT did not proceed as smoothly as desired. One difficulty in this step was the lack of solubility of the trisoxime in methylene chloride which made the addition of the material to nitric acid difficult. The initial contact between the suspension of the trisoxime and the nitric acid is rather violent so the process needed to be done slowly and on a small scale. Because of the nature of the reaction and the scale on which it was performed, only moderate yields of recovered TNT were realized. Attempts to recrystallize the oily product did not yield solid. It is probable that alternative oxidants are available for this transformation and several candidates exist in the literature.[13] It is hypothesized that a significant portion of the starting material is over-oxidized during the nitric acid reflux step which dramatically impacts yield. Regardless, the majority of the material recovered was in fact TNT. These results demonstrate proof of concept that an alternative synthetic pathway to TNT exists that might eventually circumvent conventional nitration methods and the attendant red water production.

CONCLUSIONS and IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION

All of the proposed objectives for this project have been successfully completed. TATB was synthesized from bioderived mono-, di-, and tri-*O*-methylphloroglucinol (MR, DMP, and TMB respectively) in yields that are competitive with the previously reported TATB syntheses. Nitration of the starting aromatics was achieved with an inorganic nitrate salt dissolved in sulfuric acid. Subsequent methylations were carried out in toluene using two mol-equivalents (per –OH) of trimethyl orthoformate to convert the trinitro derivatives of MR and DMP to 1,3,5-trimethoxy-2,4,6-trinitrobenzene. This material was then dissolved in toluene and treated with ammonia in a pressure reactor to afford TATB. It was found that under the amination conditions employed, the recovered TATB was ~90% pure. A supplemental particle size and morphology study was conducted that strongly suggests further manipulation of the amination conditions could potentially improve TATB purity, thereby making MR, DMP, and TMB legitimate bioderived starting materials. This work displays new synthetic routes to TATB that reduce the amount of alkylating agent by 33% (MR), 66% (DMP), and 100% (TMB) respectively for the three *O*-methylphloroglucinol derivatives (compared to phloroglucinol). Theoretically, the ideal starting material for TATB synthesis is TMB because it would completely eliminate the alkylation step. The limiting factor to this concept is the lower yields obtained from the initial nitration of TMB compared to MR and DMP. Additional research devoted toward improving the yields of this nitration reaction would be worthwhile to increase atom efficiency. The use of ethyl acetate as an extractant and reaction solvent for the nitration and amination reaction respectively demonstrated an innovative approach to decreasing the hazards associated with handling TATB. Additional effort should be put forth to optimize the transformations in ethyl acetate so as to take full advantage of the safety benefits and increased atom efficiency offered by this type of solution phase processing.

Treatment of bioderived 2-methylphloroglucinol with aqueous hydroxylamine afforded 2,4,6-trioxime-methylcyclohexane. Refluxing this material in concentrated nitric acid yielded crude TNT. The process demonstrates a non-nitrative method of synthesizing TNT that eliminates the problematic red water issues. Additional research could be devoted to this process to increase product yields by exploring alternative oxidizers. Potentially interesting, and somewhat benign, reagents for this purpose include $NaBO_3$ or MgO in acetic acid.

ACKNOWLEDGMENTS

Thanks to David Rosenberg, Rachel Newell, Michael Wright, and Alex Paraskos, all of ATK Space Systems, for their direct technical contributions to this work. The analytical chemistry department at ATK Space Systems is gratefully acknowledged for their assistance with this research. Thanks to Mike Oates of ATK Space Systems for acquiring the SEM images; and thanks to Jeffrey Deschamps of the Naval Research Laboratory, Washington DC for obtaining the single crystal X-ray structures (Appendix III).

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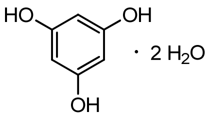
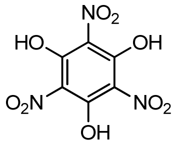
APPENDICES of ATK REPORT

Appendix I. Graphical representation of selected molecules referenced in this report.

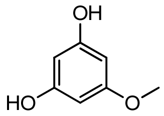
Common Name(s)
IUPAC Name



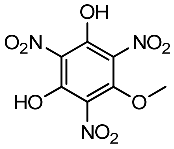
phloroglucinol dihydrate
1,3,5-trihydroxybenzene dihydrate



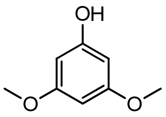
trinitrophloroglucinol
1,3,5-trihydroxy-2,4,6-trinitrobenzene



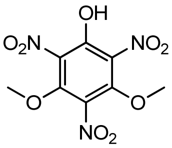
mono-*O*-methylphloroglucinol
5-methoxyresorcinol



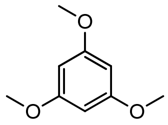
mono-*O*-methyl-trinitrophloroglucinol
5-methoxystyphnic acid
5-methoxy-2,4,6-trinitoresorcinol



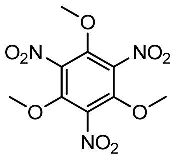
di-*O*-methylphloroglucinol
3,5-dimethoxyphenol



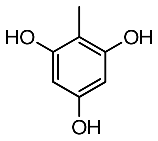
di-*O*-methyl-trinitrophloroglucinol
3,5-dimethoxypicric acid
3,5-dimethoxy-2,4,6-trinitrophenol



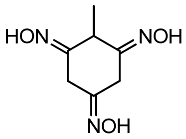
tri-*O*-methylphloroglucinol
1,3,5-trimethoxybenzene



tri-*O*-methyl-trinitro-phloroglucinol
1,3,5-trimethoxy-2,4,6-trinitrobenzene

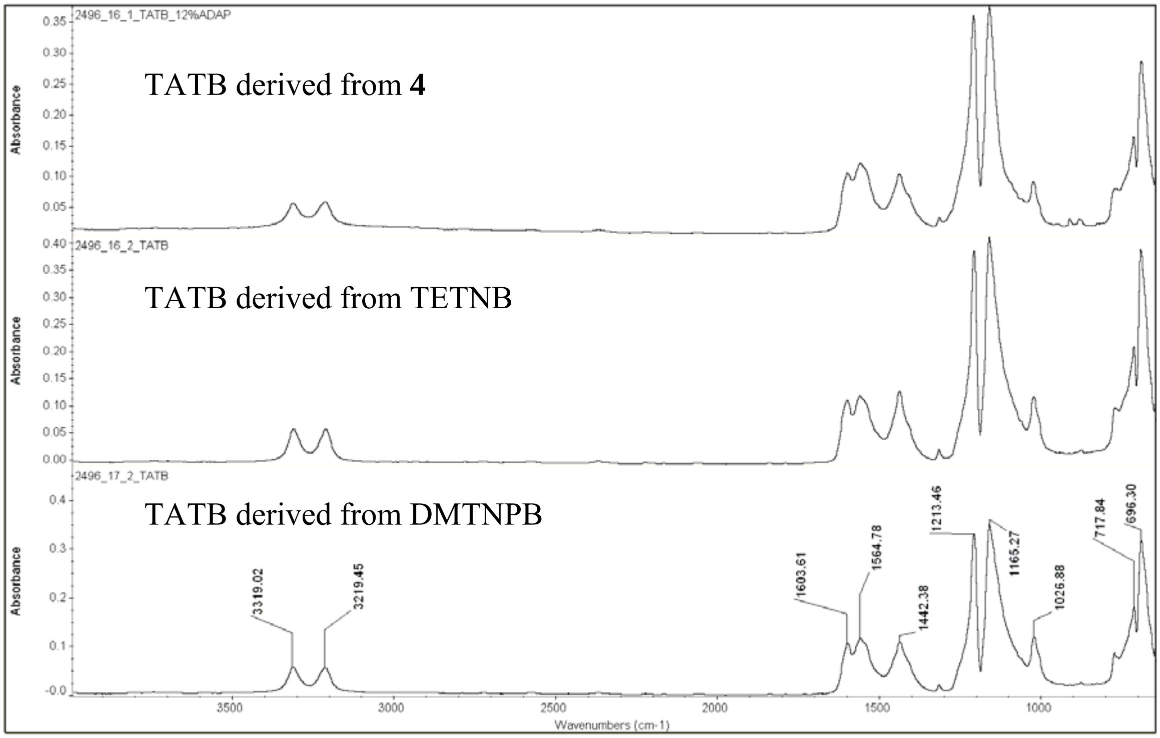


2-methylphloroglucinol
2,4,6-trihydroxytoluene



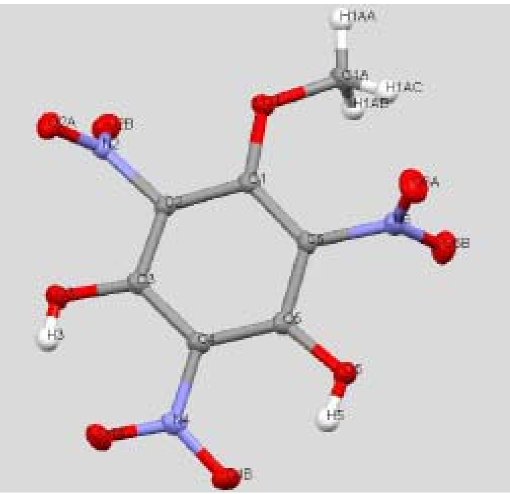
2-methylphloroglucinol trisoxime
2,4,6-trioxime-methylcyclohexane
2-methylcyclohexane-1,3,5-trione trioxime

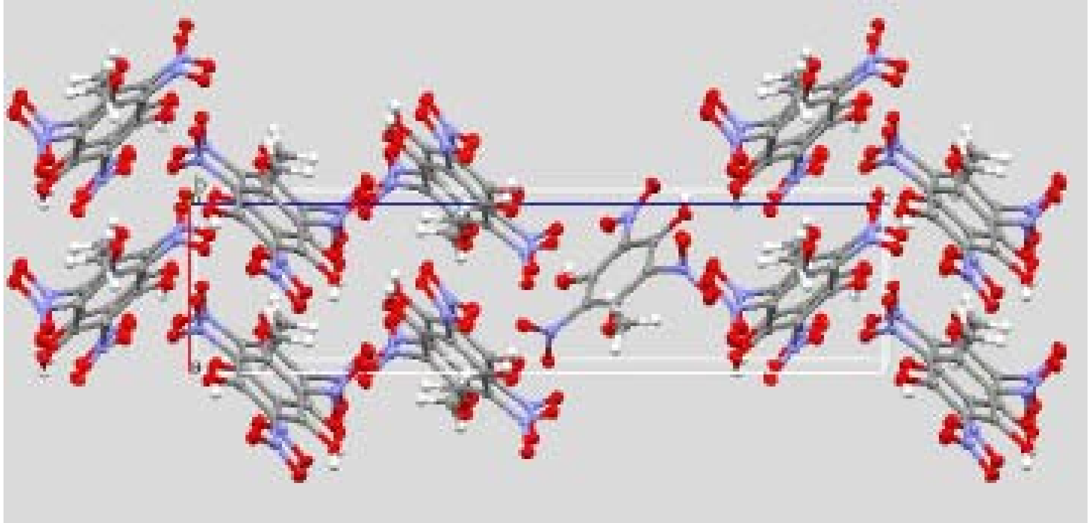
Appendix II of ATK Report. FT-IR spectra of TATB



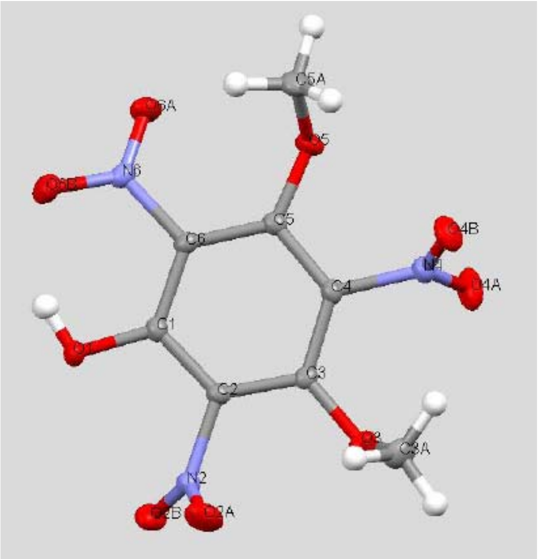
Appendix III of ATK Report. Graphical depiction of single crystal X-ray structures of **2**, **3**, and **4** courtesy of Dr. Jeffrey Deschamps, Naval Research Laboratory, Washington DC. Full structure reports are currently in preparation.

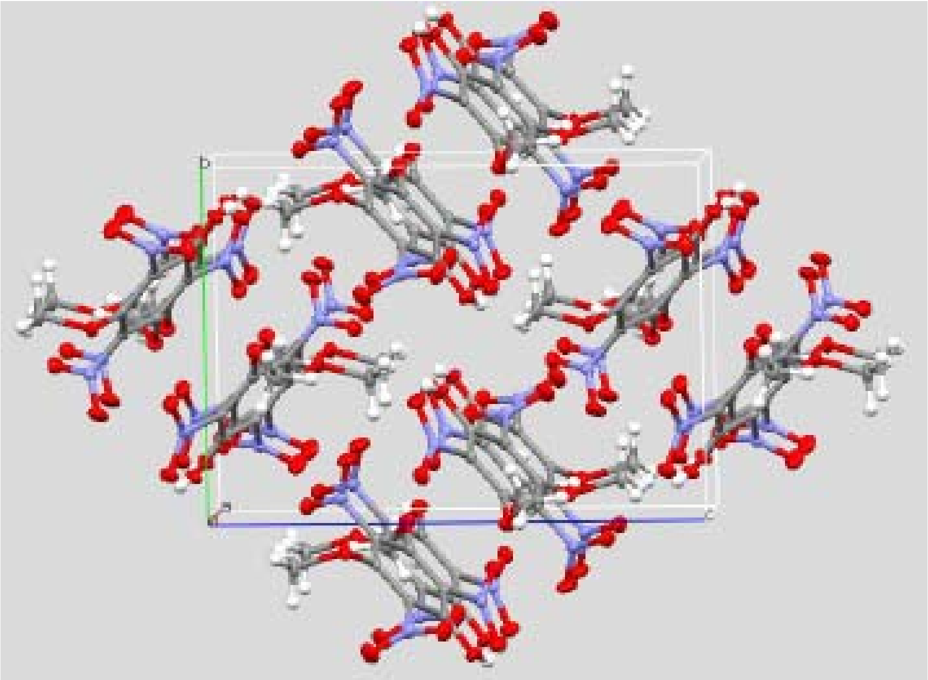
5-methoxystyphnic acid, **2**



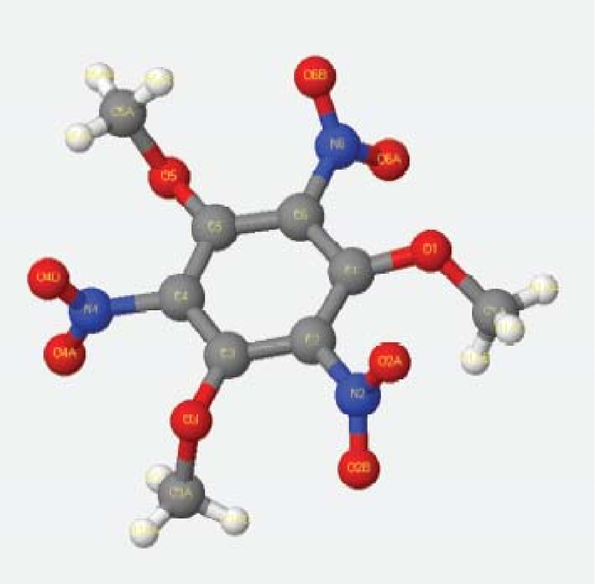


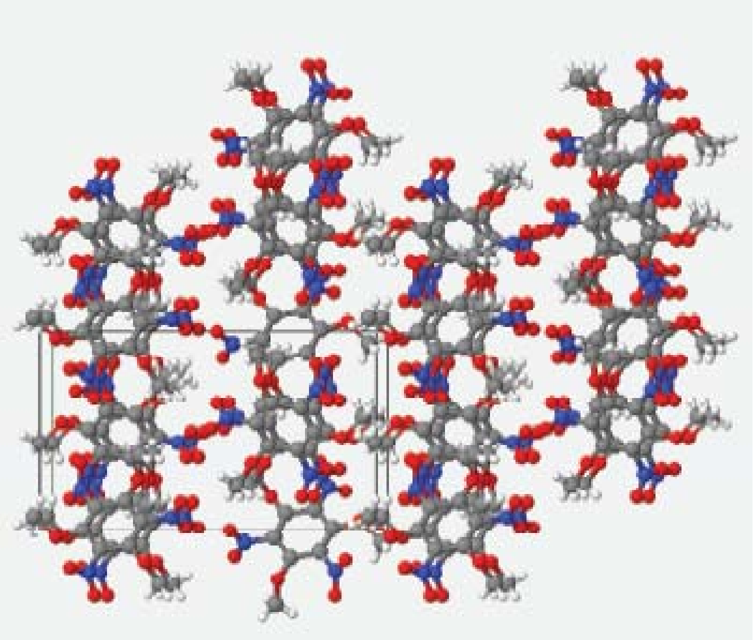
3,5-dimethoxypicric acid, **3**





1,3,5-trimethoxy-2,4,6-trinitrobenzene, **4**





Appendix IV of the ATK Report. Patent Applications, Publications, and Presentations

Patent Applications:

Straessler, N. A. *Methods for Nitrating Compounds*. Submitted 6/09

Straessler, N. A.; Velarde, S. P. *Methods of Producing 1,3,5-triamino-2,4,6-trinitrobenzene*. Submitted 6/09

Publication:

Straessler, N. A. *Synthesis of Trinitroaromatics Using Alternative Mixed Acid Nitration Conditions*. Submitted 10/09

Full single crystal X-ray structure reports of **2**, **3**, and **4** are in preparation for publication.

Poster Presentations:

Straessler, N. A. *Nitration of O-Methylated Phloroglucinols as Precursors to TATB,* Gordon Research Conference - Energetic Materials; Tilton, New Hampshire; June 6, 2008

Straessler, N. A., Frost, J. W. *Minimizing the Use of Organic Reagents in the Synthesis of TATB*, The Partners in Environmental Technology Technical Symposium and Workshop; DoD-SERDP; Washington DC; December 2-4, 2008.

Straessler, N. A. *Synthesis of Trinitrobenzene Derivatives for Applications in Energetic Materials,* 237th American Chemical Society National Meeting & Exposition, March 22-26, 2009, Salt Lake City, UT