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Discovery of a natural thiamine adenine nucleotide

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Several important cofactors are adenine nucleotides with a vitamin as the catalytic moiety. Here, we report the discovery of the first adenine nucleotide containing vitamin B1: adenosine thiamine triphosphate (AThTP, 1), or thiaminylated ATP. We discovered AThTP in *Escherichia coli* and found that it accumulates specifically in response to carbon starvation, thereby acting as a signal rather than a cofactor. We detected smaller amounts in yeast and in plant and animal tissues.

Until now, only three natural thiamine phosphate derivatives (the mono-, di- and triphosphates) were known to be present in most cells[1], and of these only thiamine diphosphate (ThDP, 2) has a well-known cofactor role[2]. We previously showed that *E. coli* accumulate thiamine triphosphate (ThTP, 3) when grown in minimal medium supplemented with a carbon source such as glucose[3] (4) (**Fig. 1a**). However, in the absence of a carbon source no ThTP was detected, and instead we noticed the appearance of an additional peak eluted after ThTP (**Fig. 1b**).

We purified this compound using different chromatographic steps (**Supplementary Table 1** online). We checked the purity by HPLC (**Supplementary Fig. 1** online), and then we collected the peak, lyophilized it and used it for mass spectrometry and ^{1}H NMR.

High-resolution ESI-FT-ICR MS gave an *m/z* of 754.097486, which corresponds to the formula $C_{22}H_{31}N_9O_{13}P_3S^+$ (theoretical monoisotopic mass of 754.096939) at sub-p.p.m. mass accuracy (**Supplementary Fig. 2** online). ESI-MS/MS fragmentation (**Supplementary Fig. 2**) suggested the presence of an AMP moiety (*m/z* of 348.1). These data are in agreement with a structure containing a thiamine moiety and an adenosine moiety linked by three phosphates—that is, adenylated ThDP (AThTP) or thiaminylated ATP (**Fig. 1c**). We confirmed the presence of the thiamine moiety and the adenosine moiety by ^{1}H NMR. Comparison with the spectra of commercial 5′-AMP (5) and 3′-AMP (6) indicated that the 5′-hydroxyl group rather than the 3′-hydroxyl group is substituted in AThTP (**Supplementary Fig. 3** online).

We further confirmed the structure of AThTP through chemical synthesis by condensation of ThDP and 5′-AMP using dicyclohexylcarbodiimide as an activator[4] (**Supplementary Methods** online). We purified the synthesized compound as described for the natural product and confirmed the identity with AThTP isolated from bacteria by high-resolution ESI-FT-ICR MS (*m/z* = 754.096862), tandem ESI-MS/MS (**Supplementary Fig. 2**) and ^{1}H NMR (**Supplementary Fig. 3**). The ^{13}C NMR spectrum of the synthetic compound (**Supplementary Fig. 3**) confirmed the proposed structure of AThTP. The purified natural AThTP and the chemically synthesized AThTP were hydrolyzed at the same rate by an enzyme present in a bacterial membrane fraction (**Supplementary Fig. 4** online), thereby confirming their identity.

When *E. coli* were incubated in minimal medium devoid of any carbon source, AThTP gradually accumulated and reached a maximum concentration after 4 h (**Fig. 2a**), at which point it accounted for 10–15% of total thiamine in the cells. Subsequent addition of glucose (10 mM) induced a rapid disappearance of AThTP followed by a transient accumulation of ThTP (**Fig. 2b**). When malate (7) was added instead of glucose, the kinetics of AThTP disappearance were slower (half-time ~ 20 min), and, in agreement with previous results[3], no ThTP was formed. When the bacteria were directly

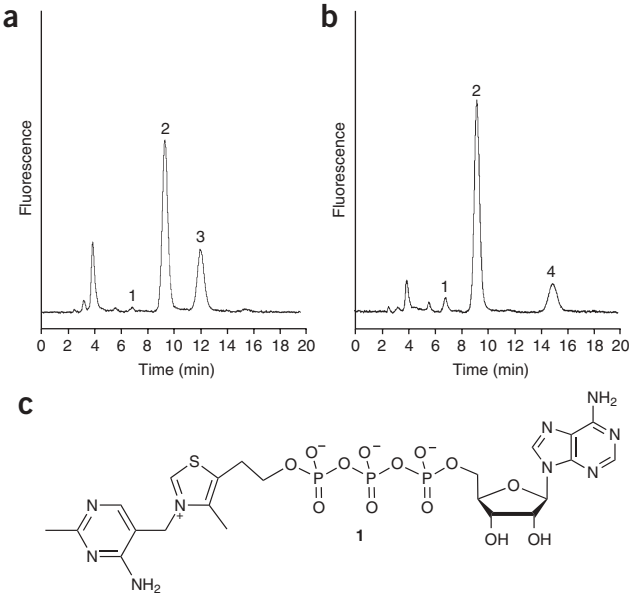

Figure 1 Chromatograms showing the presence of ThTP or the additional peak, according to culture conditions. The bacteria were grown overnight in LB medium and suspended in minimal M9 medium (**Supplementary Methods**). (**a**,**b**) They were then incubated (1 h, 37 °C) in M9 medium in the presence (**a**) or absence (**b**) of 10 mM glucose. Thiamine derivatives were determined by HPLC after oxidation to fluorescent thiochrome derivatives[8] (1, ThMP; 2, ThDP; 3, ThTP; 4, additional peak or AThTP). (**c**) Structure of AThTP.

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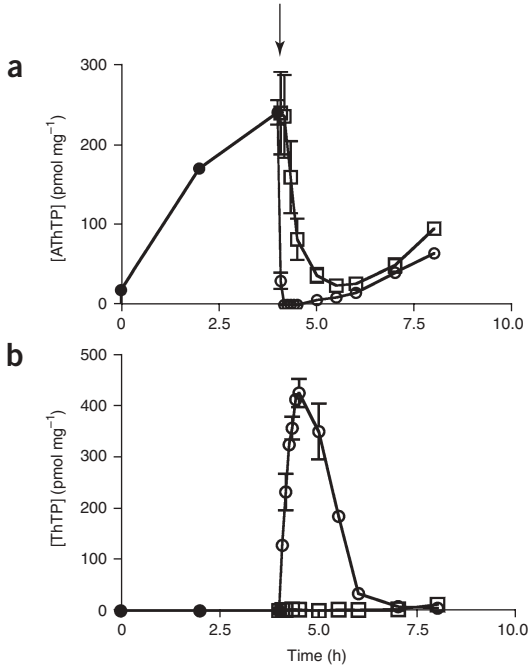

Figure 2 Effect of substrate addition on the content of AThTP and ThTP in *E. coli* BL-21 cells. (**a**,**b**) The bacteria were first incubated at 37 °C in M9 medium without a carbon source (●), and the concentrations of AThTP (**a**) and ThTP (**b**) were determined. After 4 h, glucose (○) or malate (□) was added at a final concentration of 10 mM (arrow). The results are expressed as mean ± s.d. for three experiments.

transferred from the LB medium to the minimal medium supplemented with glucose (10 mM), no significant amount of AThTP was formed. With other energy substrates such as pyruvate (8), lactate (9) and malate, AThTP concentrations also remained insignificant (**Supplementary Table 2** online).

These results suggest that AThTP is accumulated specifically in response to carbon starvation. This response was not appreciably modified by the presence or absence of a phosphate or nitrogen source (**Supplementary Table 2**). This specificity for carbon starvation differs from the synthesis of guanosine tetraphosphate (10) and guanosine pentaphosphate (11), which is known to trigger the so-called stringent response when the growth medium becomes deficient in either amino acids, carbon, phosphorus or nitrogen[5,6]. ThTP and AThTP seem to be more specific to particular types of stress: ThTP is produced in response to amino acid starvation, and a carbon source is required for its accumulation[3]. In contrast, AThTP specifically appears in response to a lack of any carbon source. Note that we never observed the simultaneous accumulation of ThTP and AThTP in large amounts, and both compounds were absent under most normal conditions of growth, such as in the presence of amino acids or in rich LB medium. Moreover, ThTP accumulation was always transient[3], whereas AThTP remained present as long as the cells were carbon starved (**Fig. 2a**).

AThTP is also present in eukaryotic cells. We found AThTP in yeast, in the roots of higher plants and in several animal tissues (**Supplementary Table 3** online). In the rat, it was present in most organs tested; the highest concentrations were found in the liver, heart, kidney and lung, and the lowest in skeletal muscle and brain. AThTP, much like ThTP, was present at much lower concentrations in eukaryotic organisms than in *E. coli*. It is possible that both compounds accumulate only in specific cells or organelles, under conditions that have not yet been defined in eukaryotes.

Among all the nucleotides found in living cells, adenine nucleotides are the most abundant and diverse. In coenzyme A (12), FAD (13), NAD^+ (14) and $NADP^+$ (15), the catalytic moiety is a vitamin: pantothenic acid, riboflavin or nicotinamide. Here, we report the discovery of the first nucleotide containing thiamine. Recently, an ADP-containing intermediate in the biosynthesis of the thiamine thiazole was described in eukaryotes[7]. The choice of the name adenosine thiamine triphosphate rather than thiamine ATP emphasizes its apparent close metabolic relationship to ThTP. The present data suggest that, at least in *E. coli*, AThTP acts as a signal rather than as a cofactor. Our findings highlight the diversity of thiamine biochemistry.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

L.B. is the project leader and performed purification of the bacterial AThTP and chemical synthesis and purification of AThTP. B.W. discovered the AThTP peak on the chromatograms, performed part of the experiments with bacteria and started the purification. A.F.M. helped to identify AThTP and studied its enzymatic synthesis and hydrolysis. G.M. and E.P. performed MS experiments. M.F and L.A. performed NMR experiments. T.G. performed part of the experiments with bacteria. M.G. measured AThTP in animal tissues. P.W. helped to design the experiments and write the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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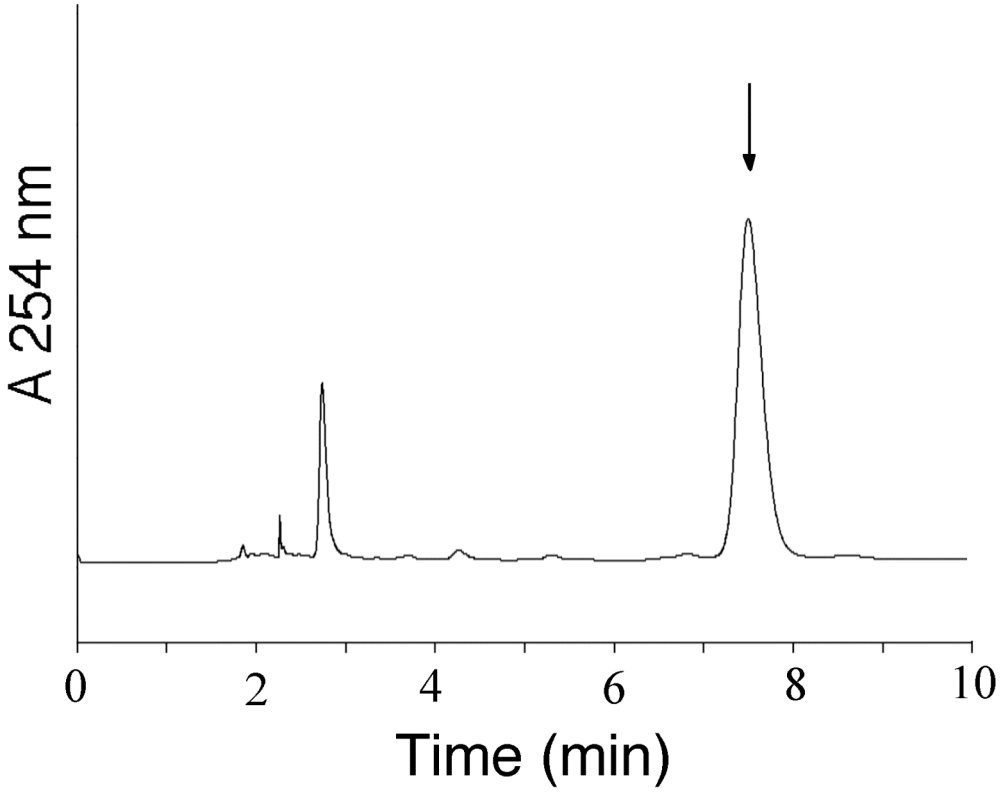
Supplementary Methods

Determination of thiamine compounds and adenine nucleotides by HPLC. We analyzed thiamine compounds as previously described after transformation into fluorescent thiochrome derivatives using a PRP-1 column (Hamilton)[7]. AThTP and other adenosine derivatives were also quantified using UV detection (254 nm) after separation on a 5 µm Chromsphere C18 column (150 x 4.6 mm, Varian). The mobile phase contained 25 mM tetra-*n*-butylammonium hydrogen sulfate, 50 mM NaH_2PO_4 adjusted to pH 7.0 and 15 % methanol. The flow rate was 1 ml/min.

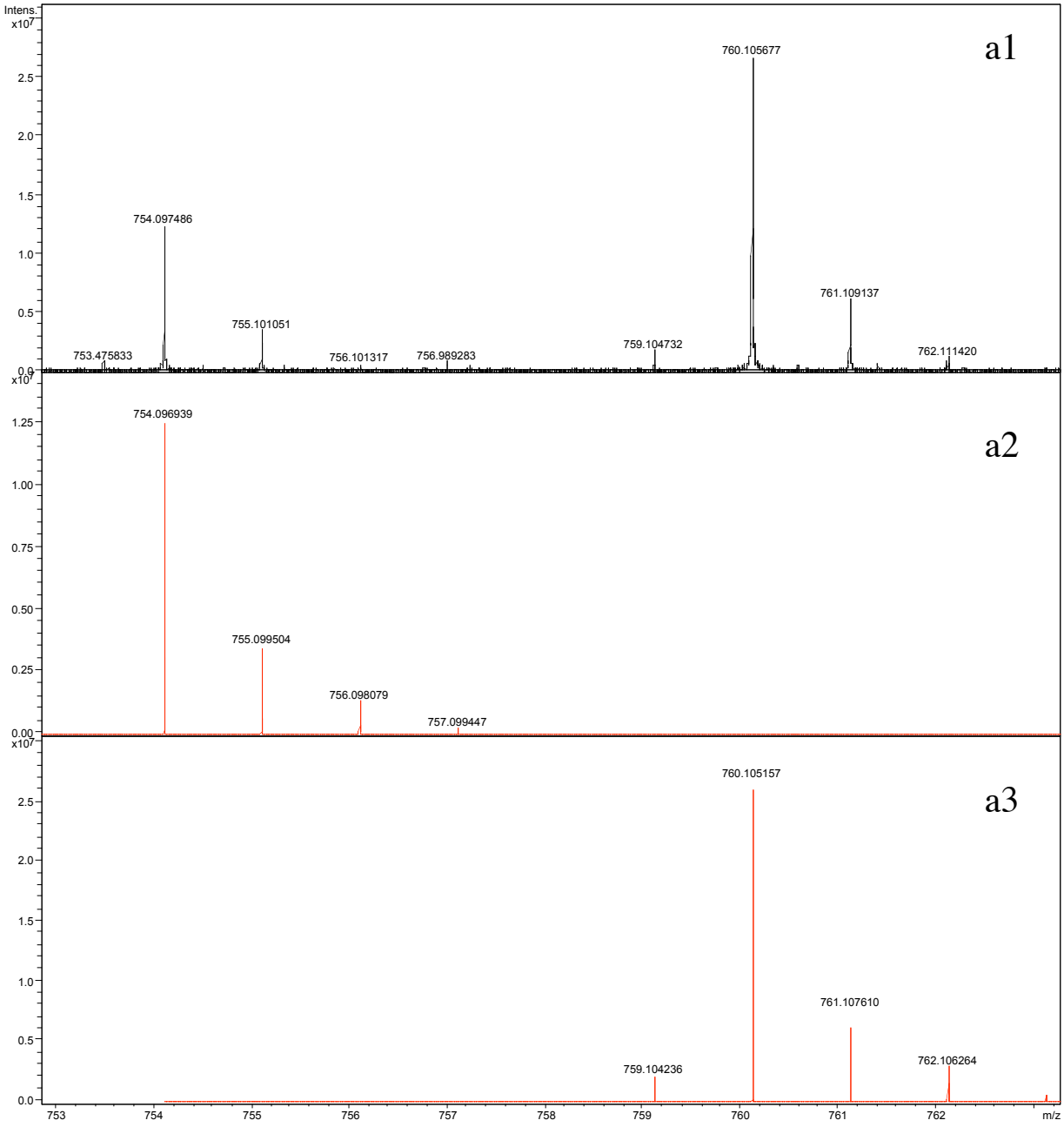
Growth and processing of bacteria. We grew *E. coli* (BL 21 or MG1655 wild-type K-12) overnight (37 °C, 250 rpm) in 50-100 ml LB medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l at pH 7.0). The bacteria were centrifuged (5 min; 10,000 x g) and suspended in the initial volume of fresh LB medium or M9 minimal medium (Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l; $CaCl_2$, 3 mg/l; $MgSO_4$, 1 mM, pH 7.0) containing various metabolic substrates. After incubation at 37 °C, the bacteria were sedimented as above, the pellets were suspended in 12% (w/v) trichloroacetic acid, the precipitated proteins spun down (15 min, 15,000 x g) and the pellet dissolved in 0.8 N NaOH for protein determination (Peterson, G.L. *Anal. Biochem.* 83, 346-356, 1977). The supernatant was treated with diethyl ether and analyzed by HPLC[7].

Chemical synthesis of AThTP. We synthesized AThTP by a modification of a previously published method[4] for the synthesis of ThTP and nucleoside triphosphates. Here, ThDP and 5'-AMP (instead of ThDP and H_3PO_4) were used as precursors. Briefly, we mixed 1.35 mmol ThDP, 2.1 mmol 5'-AMP (acid form), 2.1 ml tributylammonium and 1.5 ml H_2O until we obtained a translucent solution. We added this solution to a mixture of 300 ml dimethyl sulfoxide and 267 ml pyridine. The synthesis was initiated by addition of 30 ml dicyclohexylcarbodiimide (0.9 g/ml in pyridine). After 1 h, we added 1800 ml of diethyl ether, and the precipitate was centrifuged (1000 x g, 10 min) and dissolved in 50 ml H_2O. We purified the synthesized compound (0.44 mmol) on the AG 50W-X8 and AG-X1 resins (Bio-Rad) as described for bacterial AThTP (**Supplementary Table 1** online). We further purified approximately 33 µmol (~ 25 mg), by successive 100-µl injections on the Polaris C18 column (Varian) for ^{1}H-NMR and ^{13}C-NMR.

Supplementary Figure 1 Purification of AThTP on a Polaris C18 HPLC column. 100 µl fractions of the partially purified compound (see **Supplementary Table 1** online) were injected and eluted in 50 mM ammonium acetate buffer (pH 7.0) in 5% methanol and 1 ml fractions were collected. The fractions were checked for the presence of AThTP by HPLC after derivatization to fluorescent compounds. It was eluted between 7 and 8 min (arrow). The corresponding fractions from 10 injections were pooled, lyophilized and analyzed by mass spectrometry and ^{1}H-NMR.



Supplementary Figure 2 High resolution positive ion ESI-FT-ICR mass spectrum of AThTP isolated from bacteria (**a1**). Theoretical isotopic distribution of $C_{22}H_{31}N_9O_{13}P_3S^+$ (**a2**) and $C_{22}H_{30}N_9O_{13}P_3SLi^+$ (**a3**). High resolution positive ion ESI-FT-ICR mass spectrum of chemically synthesized AThTP (**b1**). Theoretical isotopic distribution of $C_{22}H_{31}N_9O_{13}P_3S^+$ (**b2**) and $C_{22}H_{30}N_9O_{13}P_3SLi^+$ (**b3**). Positive ion ESI mass spectrum of AThTP isolated from bacteria (**c**). AThTP was diluted at a concentration of 150 μM in H_2O: acetonitrile (50% : 50%) containing 1 mM lithium iodide. The ESI-MS/MS fragmentation pattern for AThTP isolated from *E. coli* is shown in (**d**). Positive ion ESI mass spectrum of chemically synthesized AThTP (**e**), diluted at a concentration of 150 μM in H_2O : acetonitrile (50% : 50%) and the ESI-MS/MS fragmentation pattern (**f**).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755.101051
753.475833
756.101317
756.989283
759.104732
762.111420
x10^7
a2
754.096939
755.099504
756.098079
757.099447
x10^7
a3
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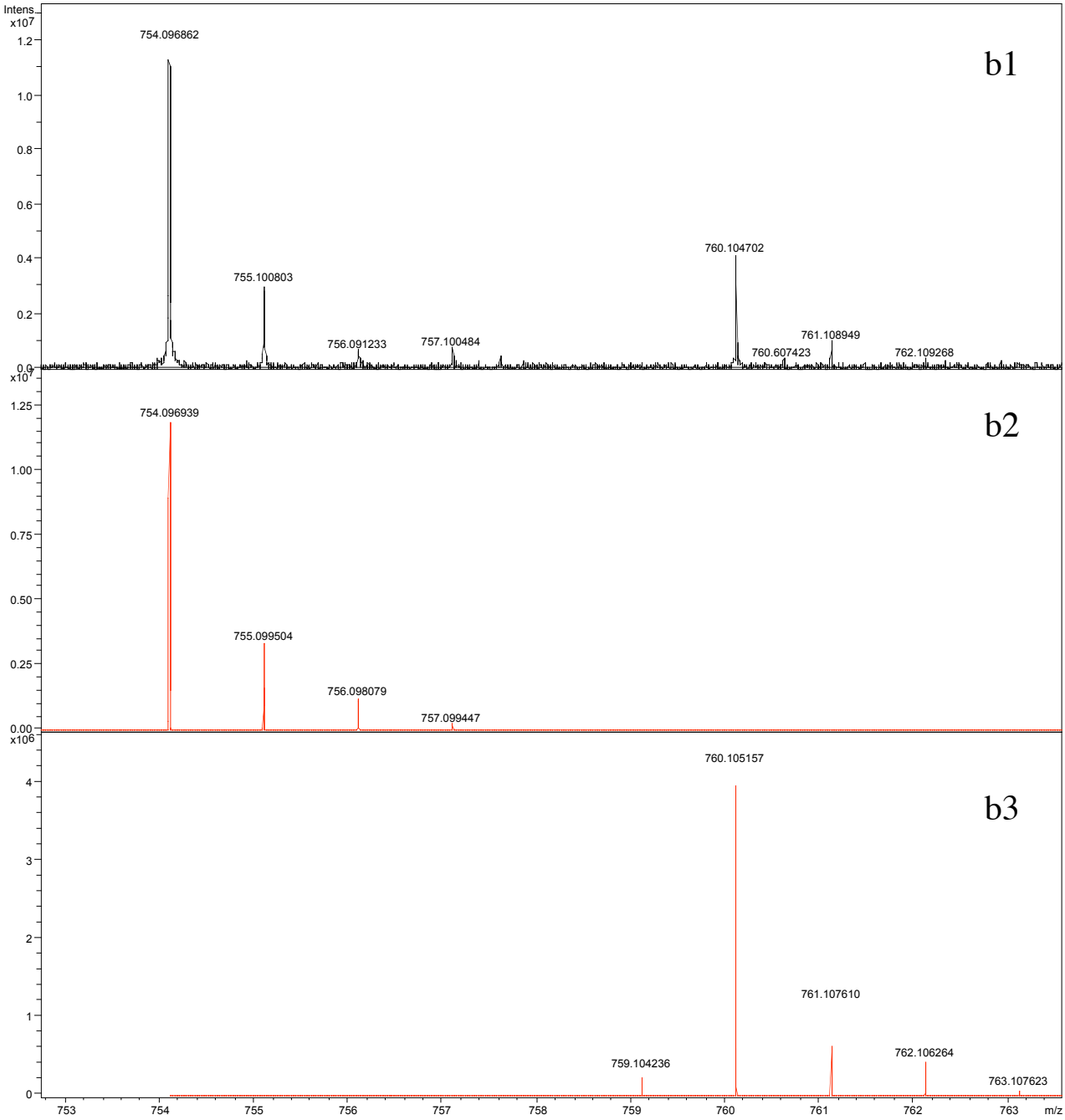
761
762
m/z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0.6
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757.100484
760.104702
760.607423
761.108949
762.109268
b1
x10^7
1.25
1.00
0.75
0.50
0.25
0.00
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756.098079
757.099447
b2
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3
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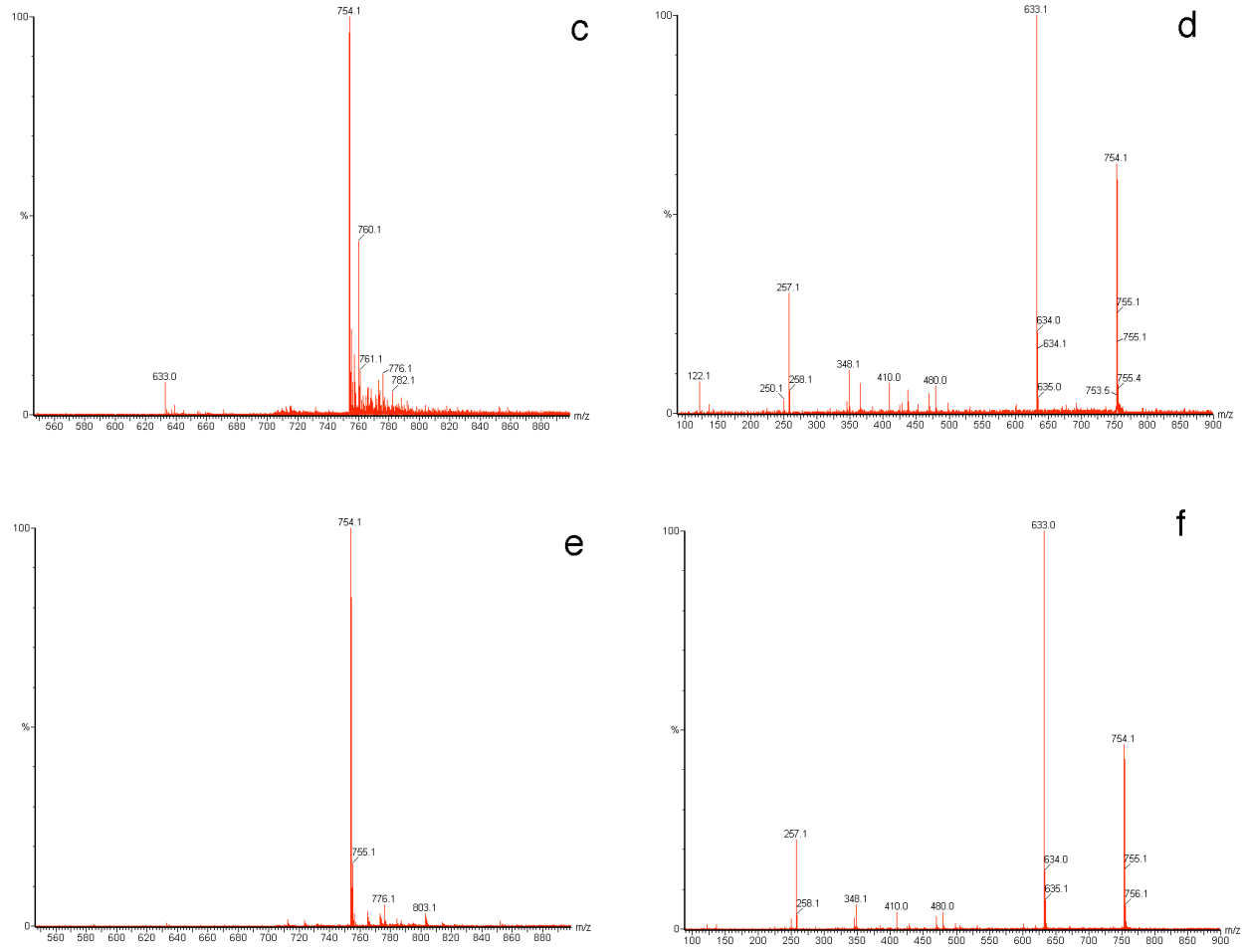


AThTP analysis by electrospray tandem mass spectrometry. Experiments were performed on a Micromass Q-TOF Ultima Global apparatus (Micromass, now Waters Corporation, Manchester, UK) operated in nano-ESI positive ion mode. The synthesized AThTP was injected at a concentration of 200 µM in 50% water - 50% acetonitrile. Lithium iodide was added at a final concentration of 1 mM to the solution containing the purified AThTP from *E. coli* to suppress the multiple undesired salt adducts. The source parameters were: capillary voltage: 1.8 kV, cone voltage: 100 V, RF lens 1: 90 V, source temperature: 80 °C, collision energy: 6 eV. The fragmentation pattern of the mass 754.1 was obtained with 30 V acceleration voltage. The major product ions were of *m/z* 633.1, 348.1 and 257.1. The 633.1 peak might arise from fragmentation at the level of the quaternary nitrogen of the thiazole moiety of thiamine with loss of the pyrimidinium part (M^+ – 121 – pyrimidinium), while the 348.1 ion might correspond to AMP.

Determination of the exact mass of AThTP. Experiments were performed on an ESI-FT-ICR mass spectrometer (Apex Qe 9.4 T, Bruker Daltonik GmbH, Bremen Germany) operated in positive ion mode. The mass spectrometer was calibrated with 0.1% phosphoric acid – 50% water – 50% acetonitrile solution in the range of the mass to be measured and gave a standard deviation accuracy of 0.284 ppm over eight calibration points. The synthesized and natural AThTP were injected separately at a concentration of 20 µM in 50% water – 50% acetonitrile containing 20 µM lithium iodide. External calibration was applied. The source parameters were: capillary voltage: 4.48 kV, capillary exit voltage: 60 V, desolvatation gas temperature: 200 °C. Molecular formula were generated (see below) using the following parameters: relative mass tolerance: 1 ppm, minimum elements: H_{30} (based on ^{1}H-NMR), maximum element: $C_{\infty}H_{31}N_{\infty}O_{\infty}P_5S_5Li$, nitrogen rule applied.

Molecular formula generated for the natural compound (M^+, M-H Li^+).

M^+

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 22 H 31 N 9 O 13 P 3 S 1	0.029	754.096939	-0.73	-1.20	-0.55	13.50	ok	even
C 24 H 31 Li 1 N 1 O 22 P 2	0.038	754.096750	-0.98	-0.88	-0.74	10.50	ok	even
C 28 H 30 N 5 O 14 P 2 S 1	0.046	754.097971	0.64	0.29	0.49	17.50	ok	even
C 25 H 30 Li 1 N 13 O 1 P 5 S 2	0.061	754.098032	0.72	-0.05	0.55	19.50	ok	even
C 32 H 30 Li 1 N 7 O 3 P 5 S 1	0.064	754.097347	-0.18	-0.48	-0.14	23.50	ok	even
C 38 H 31 N 1 O 8 P 3 S 1	0.087	754.097774	0.38	0.19	0.29	25.50	ok	even
C 23 H 31 Li 1 N 11 O 6 P 2 S 4	0.090	754.097139	-0.46	-1.34	-0.35	14.50	ok	even
C 36 H 30 Li 1 N 1 O 9 P 1 S 3	0.105	754.097486	-0.00	-0.44	-0.00	22.50	ok	even
C 29 H 30 Li 1 N 7 O 7 P 1 S 4	0.105	754.098171	0.91	0.06	0.69	18.50	ok	even
C 34 H 30 N 9 P 2 S 4	0.125	754.097674	0.25	-0.58	0.19	25.50	ok	even
C 41 H 30 N 3 O 2 P 2 S 3	0.130	754.096989	-0.66	-1.11	-0.50	29.50	ok	even
C 39 H 31 Li 1 N 3 O 1 P 2 S 4	0.135	754.097974	0.65	-1.17	0.49	26.50	ok	even

M-H Li^+

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e⁻
C 17 H 31 N 15 O 10 P 5	0.007	760.106091	0.54	0.09	0.41	12.50	ok	even
C 25 H 30 N 1 O 26	0.035	760.105057	-0.82	-0.86	-0.62	11.50	ok	even
C 18 H 30 N 7 O 24 S 1	0.038	760.105742	0.09	-0.46	0.06	7.50	ok	even
C 22 H 30 Li 1 N 9 O 13 P 3 S 1	0.038	760.105118	-0.74	-1.23	-0.56	13.50	ok	even
C 23 H 31 Li 1 N 1 O 25 S 1	0.043	760.106042	0.48	0.17	0.36	8.50	ok	even
C 15 H 30 Li 1 N 15 O 11 P 3 S 2	0.048	760.105803	0.17	-0.02	0.13	9.50	ok	even
C 21 H 31 N 9 O 16 P 1 S 2	0.058	760.106230	0.73	-0.05	0.55	11.50	ok	even
C 20 H 30 N 17 O 4 P 4 S 2	0.064	760.105306	-0.49	-0.53	-0.37	16.50	ok	even
C 25 H 31 Li 1 N 11 O 5 P 4 S 2	0.065	760.105606	-0.09	0.01	-0.07	17.50	ok	even
C 18 H 31 Li 1 N 17 O 3 P 4 S 3	0.066	760.106291	0.81	0.53	0.61	13.50	ok	even
C 28 H 31 N 3 O 18 P 1 S 1	0.067	760.105545	-0.17	-0.61	-0.13	15.50	ok	even
C 32 H 31 Li 1 N 5 O 7 P 4 S 1	0.074	760.104921	-1.00	-1.32	-0.76	21.50	ok	even
C 38 H 30 Li 1 N 1 O 8 P 3 S 1	0.098	760.105953	0.36	0.11	0.28	25.50	ok	even
C 22 H 31 Li 1 N 11 O 9 S 5	0.112	760.106431	0.99	0.01	0.75	12.50	ok	even
C 24 H 30 N 11 O 10 S 4	0.114	760.105445	-0.30	-0.21	-0.23	15.50	ok	even
C 29 H 31 Li 1 N 5 O 11 S 4	0.118	760.105745	0.09	0.27	0.07	16.50	ok	even
C 17 H 30 N 17 O 8 S 5	0.119	760.106131	0.60	0.31	0.45	11.50	ok	even
C 27 H 31 N 13 O 2 P 1 S 5	0.132	760.105934	0.34	-0.71	0.26	19.50	ok	even
C 34 H 31 N 7 O 4 P 1 S 4	0.134	760.105248	-0.56	-1.13	-0.43	23.50	ok	even
C 43 H 30 N 3 O 1 P 4 S 1	0.140	760.105456	-0.29	-0.48	-0.22	32.50	ok	even
C 40 H 30 N 3 O 5 S 4	0.152	760.106280	0.79	-0.75	0.60	27.50	ok	even

Molecular formula generated for the synthesized compound (M^+, M-H Li^+).

M^+

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e^-
C 19 H 30 N 7 O 21 P 2	0.030	754.096451	-0.55	-0.73	-0.41	9.50	ok	even
C 22 H 31 N 9 O 13 P 3 S 1	0.037	754.096939	0.10	-0.66	0.08	13.50	ok	even
C 24 H 31 Li 1 N 1 O 22 P 2	0.045	754.096750	-0.15	-0.02	-0.11	10.50	ok	even
C 29 H 31 N 3 O 15 P 3	0.046	754.096253	-0.81	-0.70	-0.61	17.50	ok	even
C 20 H 30 Li 1 N 9 O 14 P 1 S 3	0.068	754.096651	-0.28	-0.65	-0.21	10.50	ok	even
C 32 H 30 Li 1 N 7 O 3 P 5 S 1	0.073	754.097347	0.64	-0.02	0.48	23.50	ok	even
C 25 H 30 N 11 O 7 P 2 S 3	0.074	754.096154	-0.94	-1.25	-0.71	17.50	ok	even
C 18 H 30 N 17 O 5 P 2 S 4	0.079	754.096839	-0.03	-0.84	-0.02	13.50	ok	even
C 39 H 30 Li 1 N 1 O 5 P 5	0.086	754.096661	-0.27	0.17	-0.20	27.50	ok	even
C 30 H 31 Li 1 N 5 O 8 P 2 S 3	0.087	754.096454	-0.54	-0.60	-0.41	18.50	ok	even
C 23 H 31 Li 1 N 11 O 6 P 2 S 4	0.088	754.097139	0.37	-0.21	0.28	14.50	ok	even
C 36 H 30 Li 1 N 1 O 9 P 1 S 3	0.106	754.097486	0.83	-0.21	0.62	22.50	ok	even
C 41 H 30 N 3 O 2 P 2 S 3	0.133	754.096989	0.17	-0.52	0.13	29.50	ok	even

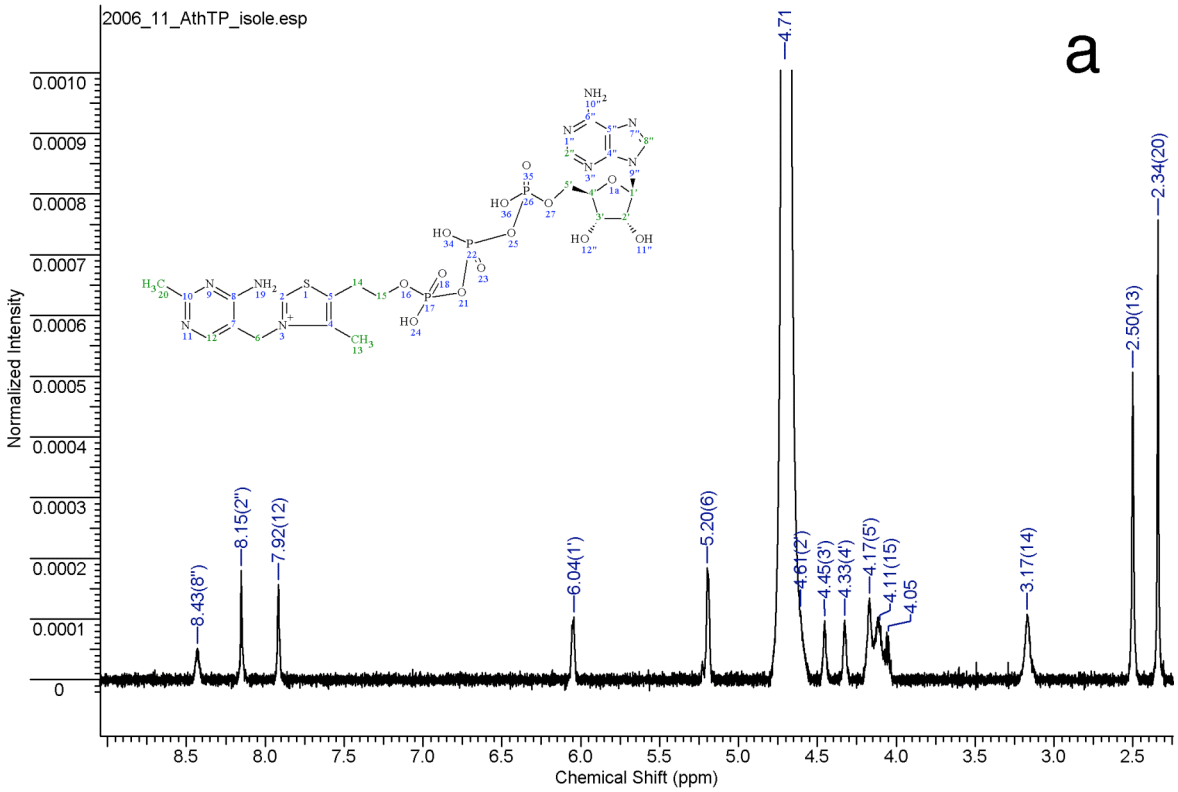
M-H Li^+

Sum Formula	Sigma	m/z	Err [ppm]	Mean Err [ppm]	Err [mDa]	rdb	N Rule	e^-
C 19 H 31 N 5 O 25 P 1	0.017	760.104025	-0.89	-1.20	-0.68	7.50	ok	even
C 25 H 30 N 1 O 26	0.019	760.105057	0.47	0.31	0.35	11.50	ok	even
C 20 H 30 N 17 O 4 P 4 S 2	0.034	760.105306	0.79	-0.51	0.60	16.50	ok	even
C 22 H 30 Li 1 N 9 O 13 P 3 S 1	0.041	760.105118	0.55	-0.22	0.42	13.50	ok	even
C 27 H 30 N 11 O 6 P 4 S 1	0.047	760.104621	-0.11	-0.91	-0.08	20.50	ok	even
C 29 H 30 Li 1 N 3 O 15 P 3	0.050	760.104432	-0.36	-0.44	-0.27	17.50	ok	even
C 20 H 31 Li 1 N 7 O 18 S 3	0.060	760.104225	-0.63	-1.69	-0.48	8.50	ok	even
C 32 H 31 Li 1 N 5 O 7 P 4 S 1	0.067	760.104921	0.29	-0.26	0.22	21.50	ok	even
C 18 H 31 N 15 O 9 P 1 S 4	0.070	760.104413	-0.38	-1.81	-0.29	11.50	ok	even
C 24 H 30 N 11 O 10 S 4	0.087	760.105445	0.98	-0.39	0.74	15.50	ok	even
C 31 H 30 N 5 O 12 S 3	0.089	760.104760	0.08	-0.90	0.06	19.50	ok	even
C 37 H 31 N 7 P 5 S 1	0.094	760.104424	-0.37	-0.97	-0.28	28.50	ok	even
C 35 H 30 Li 1 N 7 O 1 P 3 S 3	0.100	760.104136	-0.75	-1.60	-0.57	25.50	ok	even
C 34 H 31 N 7 O 4 P 1 S 4	0.109	760.105248	0.72	-0.41	0.55	23.50	ok	even
C 43 H 30 N 3 O 1 P 4 S 1	0.122	760.105456	0.99	0.47	0.75	32.50	ok	even
C 41 H 31 N 1 O 6 P 1 S 3	0.128	760.104563	-0.18	-0.99	-0.14	27.50	ok	even

Supplementary Figure 3 ^{1}H-NMR spectrum of natural AThTP (**a**) and ^{1}H-NMR (**b**) and ^{13}C-NMR (**c**) spectra of chemically synthesized AThTP. Couplings between protons 14, 15 and 1', 2', 3', 4', 5' were also observed in the COSY spectrum. NMR data are in agreement with data recorded under the same conditions for thiamine and adenosine reference samples. All data are consistent with the structural formula given in **figure 1c**. Bacterial AThTP was produced from 10 liter of culture and purified as described in **Supplementary Table 1**. A total amount of 0.4 mg of AThTP was obtained from the bacterial extract and, after purification, 50 µg remained that were used for ^{1}H-NMR.

Identification of AThTP by ^{1}H-NMR. One-dimensional ^{1}H-NMR spectra of synthetic and natural AThTP were recorded at 15 °C on a Bruker Avance 500 spectrometer operating at a proton NMR frequency of 500.13 MHz, using a 5 mm probe, 1.7 mm inserts (Bruker-Biospin) and a simple pulse-acquire sequence. Acquisition parameters consisted of a spectral width of 10330.6 Hz, a pulse width of 2.541 µs, an acquisition time of 3.17 s and a relaxation delay of 1 s. 2048 scans were recorded. FIDs were Fourier transformed with LB = 0.3 Hz and GB = 0. The resonance at 4.750 ppm due to residual solvent (HOD) was used as internal reference. A COSY spectrum was also recorded using standard Bruker parameters (cosygpqf pulse program) and 32 scans. AThTP NMR data: ^{1}H-NMR (500 MHz, deuteurion oxide) δ ppm 2.34 (s, CH3-20), 2.50 (s, CH3-13), 3.17 (br. s., CH2-14), 4.11 (m, CH2-15), 4.17 (br. s., CH2-5'), 4.33 (br. s., CH-4'), 4.45 (br. s., CH-3'), 4.62 (m, CH-2'), 5.20 (br. s., CH2-6), 6.04 (br. s., CH-1'), 7.92 (s, CH-12), 8.15 (s, CH-2''), 8.43 (s, CH-8'').

Identification of AThTP by ^{13}C-NMR. One and two-dimensional ^{13}C-NMR spectra (APT, HSQC, HMBC) of synthetic AThTP were also recorded at 15 °C on a Bruker Avance spectrometer operating at a carbon frequency of 125.03 MHz, using a 5 mm probe and standard Bruker parameters (ns = 10k for APT, ns = 128 for 2D experiments). TMS was used as internal reference. AThTP NMR data: ^{13}C-NMR (125 MHz, deuteurion oxide) δ ppm 14.1 (CH3-13), 26.0 (CH3-20), 30.4 (CH2-14), 53.6 (CH2-6), 67.7 (CH2-15), 68.2 (CH2-5'), 73.2 (CH-3'), 77.1 (CH-2'), 86.7 (CH-4'), 89.6 (CH-1'), 107.2 (C-7), 121.1 (C-5''), 138.1 (C-4), 142.4 (CH-8''), 146.1 (C-5), 151.6 (C-4''), 155.5 (CH-2''), 156.6 (CH-12), 158.0 (C-6''), 164.6 (C-8), 170.1 (C-10).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a
Normalized Intensity
Chemical Shift (ppm)
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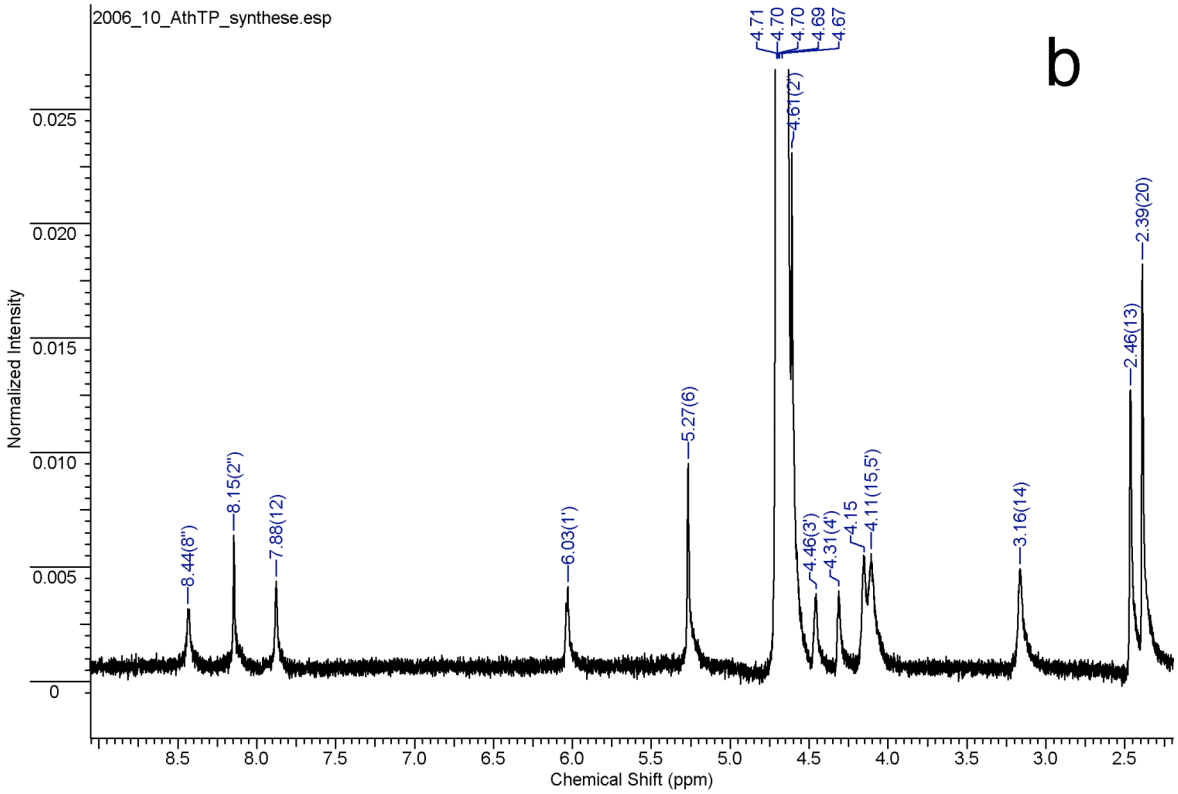
8.15(2'')
7.92(12)
6.04(1')
5.20(6)
4.71
4.61(2')
4.45(3')
4.33(4')
4.17(5')
4.11(15)
4.05
3.17(14)
2.50(13)
2.34(20)


2006_10_AthTP_synthese.esp
b
Normalized Intensity
Chemical Shift (ppm)
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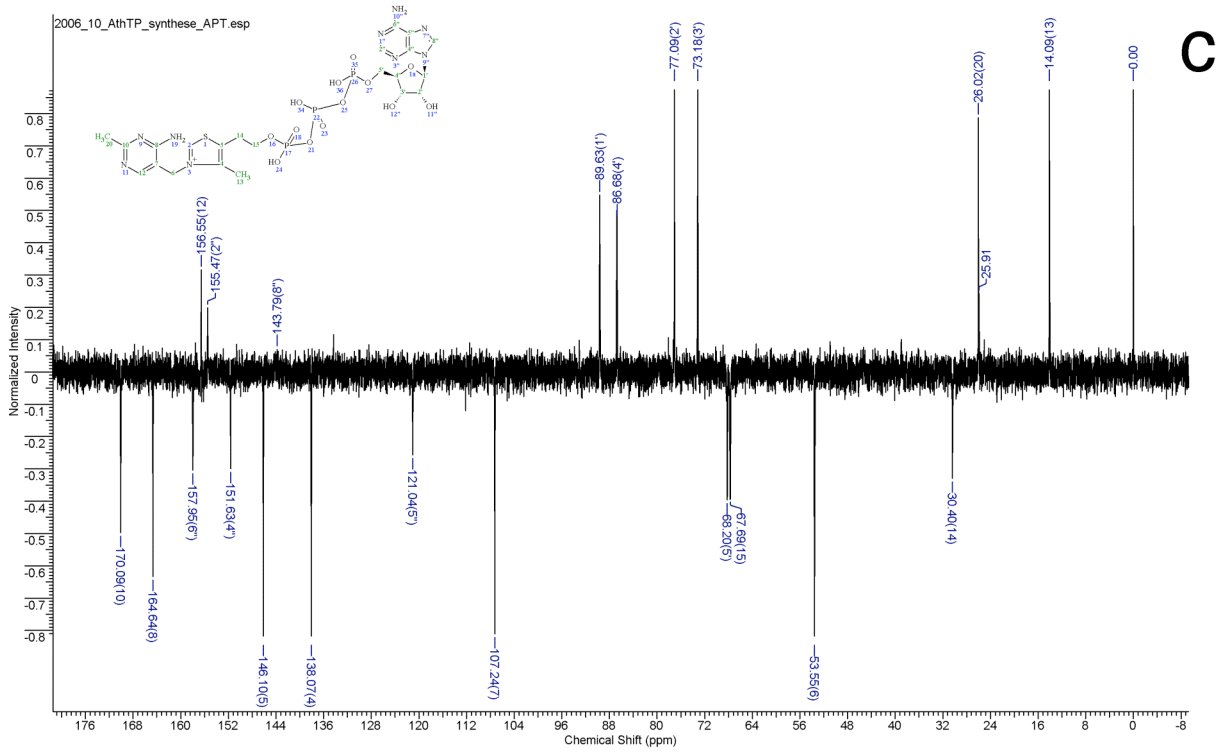
8.15(2'')
7.88(12)
6.03(1')
5.27(6)
4.71
4.70
4.70
4.69
4.67
4.61(2')
4.46(3')
4.31(4')
4.15
4.11(15,5')
3.16(14)
2.46(13)
2.39(20)

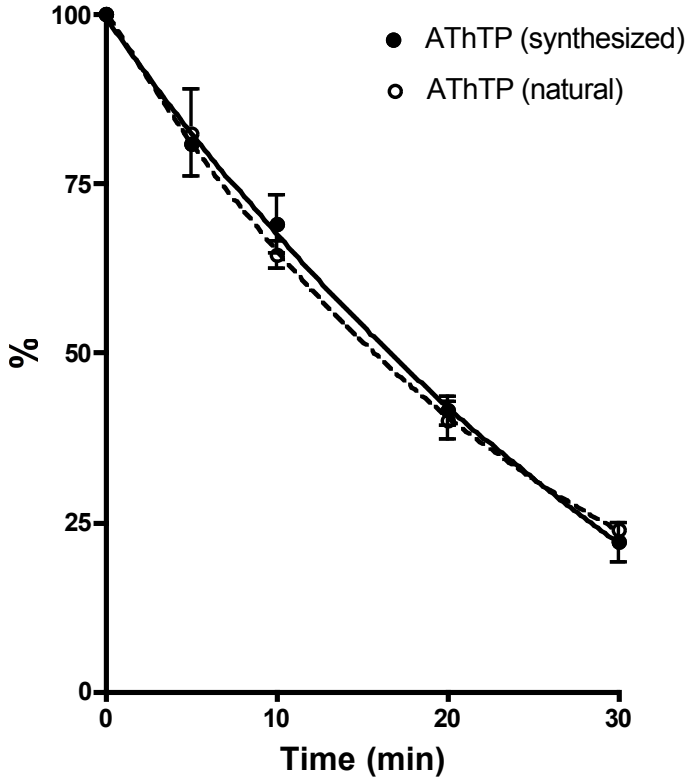


Comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts (ppm) of the 2'-, 3'- and 5'-hydroxyl groups of natural (only ^{1}H-NMR) and chemically synthesized AThTP with commercially available 5'-AMP and 3'AMP.

	AThTP (natural)	AThTP (chemical)	5'-AMP	3'-AMP
^{1}H-NMR				
2'-H	4.61[1]	4.61[1]	4.71[1]	4.86
3'-H	4.45	4.46	4.47	4.71[1]
5'-H_2	4.17	4.15	4.10	3.87
^{13}C-NMR				
2'-CH	-	77.09	77.18	75.92
3'-CH	-	73.18	73.25	76.95
5'-CH_2	-	68.20	67.22	64.17

[1] Interference with HOD peak.

Supplementary Figure 4 Rate of enzymatic breakdown of natural and chemically synthesized AThTP by a bacterial membrane fraction.



E. coli (Mg1655) grown overnight in LB medium were suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M KCl and 0.2 mM EDTA and sonicated (100 kHz, 5 x 10 s, on ice). Cell debris were sedimented (1000 x g; 10 min) and discarded. Crude membranes were collected by centrifugation (100,000 x g, 60 min) and suspended in the initial volume of Tris-HCl buffer. AThTP (0.1 mM), either purified from bacteria (○) or chemically synthesized (●) as described in **Supplementary Methods** online, was incubated for 30 min (37 °C) in the presence of 10 µl of the membrane suspension (10 mg of protein/ml), 50 mM MOPS (pH 7.2) and 10 mM $MgSO_4$ in a total volume of 100 µl. After 30 min, the reaction was stopped by addition of 100 µl trichloroacetic acid (20% w/v) and AThTP and other thiamine derivatives was determined by HPLC as described in **Supplementary Methods** online. The results are expressed as mean ± SD for three experiments.

The main products of hydrolysis were ThDP and ThMP (probably formed from ThDP by an endogenous phosphates activity). AMP was formed transiently and rapidly degraded in other products by membrane-associated enzymes.

Supplementary Table 1 Purification of AThTP from *E. coli*

Purification step	Concentration (μM)	Volume (ml)	Quantity (nmol)
Supernatant	5.9	37	218 (100)
AG 50W-X8	1.36	140	190 (87)
After lyophilization	18.8	10	188 (86)
AG-X1	0.34	280	95 (44)
After lyophilization	22	3	66 (30)
Prevail C18	21	1	21 (9,6)
Polaris C18	130	0.1	13 (6)

During purification, AThTP was estimated after oxidation, followed by HPLC analysis (**Supplementary Methods** online). The percentage yield is indicated under parentheses.

E. coli (BL 21 or MG1655) were grown overnight in 2 x 500 ml LB medium. The bacteria were centrifuged (5,500 x g, 10 min), suspended in 2 x 500 ml M9 medium without any carbon source and incubated for 3 h (37 °C, 250 rpm) for synthesis of AThTP. The bacteria were then centrifuged (5,500 x g, 10 min) and the pellets were combined and resuspended in 40 ml of 12% trichloroacetic acid. After 30 min on ice, the proteins were sedimented (10,000 x g, 15 min) and the trichloroacetic acid was extracted from the supernatant by 3 x 150 ml diethyl ether. The solution (37 ml) was then layered on a column (9 x 2.5 cm) filled with AG 50W-X8 cation exchange resin (H^+ form, Bio-Rad) in water. The column was washed with 260 ml of ice-cold water at a flow rate of 2 ml/min and 8 ml fractions were collected. AThTP was eluted with ice-cold ammonium acetate (0.2 M, pH 7.0). The fractions containing AThTP (400-540 ml) were pooled and lyophilized. The residue was dissolved in 10 ml of water and applied to a column (9 x 2.5 cm) filled with AG-X1 resin (Cl^- form, Bio-Rad) conditioned with water. The flow rate was 2 ml/min and 10 ml fractions were collected. The column was washed with 160 ml of ice-cold water, followed by 160 ml of ammonium acetate (0.25 M, pH 5.0) and then by 300 ml of ammonium acetate (0.5 M, pH 5.0). Nearly half of the AThTP was lost during this step, which is nevertheless essential to separate AThTP from the other thiamine derivatives (essentially ThMP and ThDP). The relevant fractions were pooled (280 ml) and lyophilized. The residue was dissolved in 3 ml of water and filtered on a Millex-GP filter unit (0.22 μM, ∅ 25 mm, Millipore). Aliquots of 100 μl of the pool were further purified on a Prevail C18 column (Grace) using gradient elution (0 - 20% methanol in water in 20 min) at a flow rate of 1 ml/min. All the fractions containing AThTP (retention time of

16 min) from the different runs were pooled, lyophilized and the residue dissolved in 1 ml of water. Aliquots of 100 µl of the pool were then purified on a Polaris C18 column (4.6 x 150 mm, 5 µm, Varian). The mobile phase consisted of 50 mM ammonium acetate and 5% methanol in water and the flow rate was 1 ml/min. AThTP was eluted as a symmetrical peak with a retention time of 7.5 min (**Supplementary Fig. 1** online). The peak was collected, lyophilized and used for mass spectrometry and ^{1}H-NMR.

Supplementary Table 2 Effect of media composition on the contents of ThTP and AThTP in *E. coli*

Incubation medium	ThTP (pmol/mg of protein)	AThTP (pmol/mg of protein)
Minimal M9 medium	n. d.	111 ± 11
M9 + D-glucose (10 mM)	240 ± 40	n. d.
Nitrogen-free minimal medium (a)	n. d.	79 ± 13
Nitrogen-free minimal medium + D-glucose (10 mM)	326 ± 60	n. d.
Phosphate-free minimal medium (b)	n. d.	120 ± 15
Phosphate-free minimal medium + D-glucose (10 mM)	121 ± 2	n. d.
M9 + pyruvate (10 mM)	90 ± 39	n. d.
M9 + L-lactate (10 mM)	67 ± 10	5 ± 1
M9 + L-malate (10 mM)	n. d.	n. d.
M9 + amino acids (c)	15 ± 4	n. d.

The bacteria (BL-21) were grown overnight in LB medium, suspended in minimal medium (M9 or modified as indicated) and incubated 1 h at 37 °C, 250 rpm. After incubation, the bacteria were sedimented (5 min, 10,000 x g) and the pellets were suspended in 12% (w/v) trichloroacetic acid and centrifuged. The supernatant was extracted with diethyl ether and the thiamine compounds were determined by HPLC after derivatization to thiochrome derivatives. The results are expressed as mean ± SD for 3 – 6 experiments.

n. d.: not detected (< 5 pmol/mg for ThTP and < 2.5 pmol/mg for AThTP)

M9 medium contained Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l; $CaCl_2$, 3 mg/l; $MgSO_4$, 1 mM, pH 7.0

(a) The nitrogen-free medium had the same composition as M9 medium but NH_4Cl was omitted and the NaCl concentration was 1.5 g/l

(b) The phosphate-free minimal medium contained Hepes-Na 20 mM, pH 7.0 ; NaCl, 52 mM ; KCl, 11 mM ; NH_4Cl, 1 g/l ; $CaCl_2$, 3 mg/l ; $MgSO_4$, 1 mM.

(c) The amino acid mixture contained all 20 amino acids, each at 0.5 mM except for tyrosine (0.05 mM) and tryptophan (0.1 mM).

Supplementary Table 3 Occurrence of AThTP and ThTP in eukaryotic organisms

	ThTP	AThTP
	pmol . g^{-1} of wet weight, mean ± SD, n = 3	
Arabidopsis thaliana		
Roots	n. d.	14 ± 4
Leaves	n. d.	n. d.
Parsley (*Petroselium crispum*)		
Roots	n. d.	33 ± 12
Leaves	n. d.	n. d.
	pmol . mg^{-1} of protein, mean ± SD, n = 3	
Yeast (*Saccharomyces cerevisiae*)	2.1 ± 0.3	0.23 ± 0.02
Rat (*Rattus norvegicus* Wistar)		
Brain	0.6 ± 0.2	< 0.02
Skeletal muscle	1.6 ± 0.5	0.03 ± 0.05*
Heart	< 0.05	0.6 ± 0.1
Liver	0.10 ± 0.03	0.5 ± 0.1
Kidney (cortex)	0.32 ± 0.05	0.4 ± 0.2
Kidney (medulla)	0.2 ± 0.1	0.55 ± 0.15
Spleen	0.4 ± 0.2	0.25 ± 0.10
Lung	0.3 ± 0.1	0.50 ± 0.05
Testis	0.8 ± 0.2	0.20 ± 0.05

Tissues (approximately 100 mg) were homogenized in 500 µl of 12% trichloroacetic acid in a glass-glass homogenizer and centrifuged (5,000 x g, 15 min). The supernatant was treated with 3 x 1.5 ml diethyl ether to remove the acid. The samples were then analyzed by HPLC[7]. AThTP was identified by spiking the samples with chemically synthesized AThTP. Controls were also made by injecting the samples without oxidation, conditions under which only naturally fluorescent compounds are observed but not thiochromes. Protein content was determined by the method of Peterson (Peterson, G.L. *Anal. Biochem.* **83**, 346-356, 1977). The use of animals was approved by the Institutional Committee for Animal Care and Use (#526).

n. d., not detected

* AThTP was found in only 1 of the 3 samples