Supplementary Material

How Reliable is Internal Standard Method in Monitoring Mechanochemical Synthesis? A Case Study of Triphenylmethane in HPLC-UV-MS Analysis of Hemicucurbit[*n*]urils

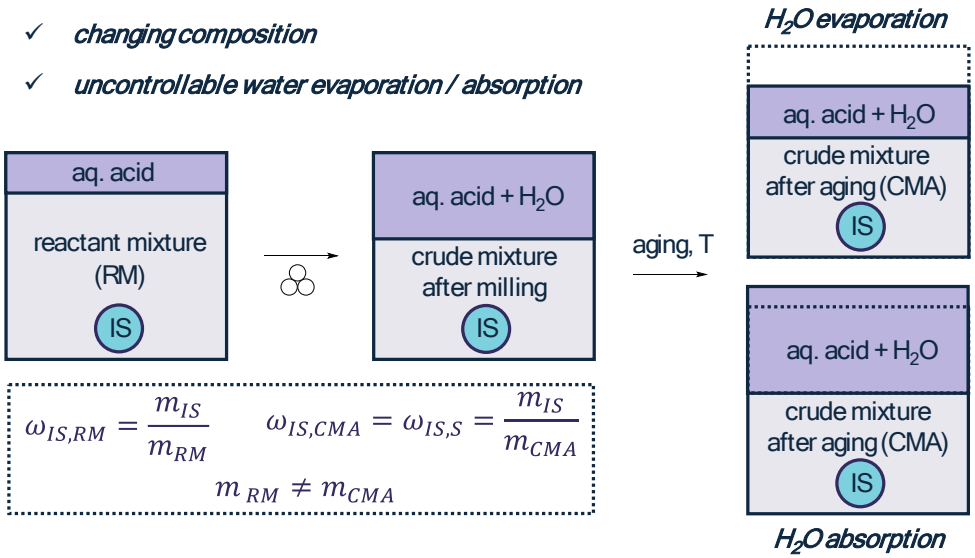
Tatsiana Jarg, Jevgenija Tamm, Elina Suut-Tuule, Ketren-Marlein Lootus, Dzmitry Kananovich and Riina Aav*

Tallinn University of Technology, School of Science, Department of Chemistry and Biotechnology, Akadeemia tee 15, 12618 Tallinn, Estonia.

*E-mail: riina.aav@taltech.ee

Table of Contents

Yield calculation



Scheme S1. Representation of the changes in the composition of reaction mixture occurring during milling and aging.

The formula for calculating the macrocycle yield, %, was derived as follows (1):

$$Yield = \frac{n_{HC} \cdot N_{mon} \cdot 100}{n_{st.mon}} = \frac{m_{RM} \cdot S_{HC} \cdot m_S \cdot V_S \cdot k_{IS} \cdot m_{IS} \cdot P_{IS} \cdot N_{mon} \cdot 100}{m_{RM} \cdot S_{IS} \cdot m_S \cdot V_S \cdot k_{HC} \cdot 100 \cdot M_{HC} \cdot n_{st.mon}} = \frac{S_{HC} \cdot k_{IS} \cdot m_{IS} \cdot P_{IS} \cdot N_{mon}}{S_{IS} \cdot k_{HC} \cdot M_{HC} \cdot n_{st.mon}} \quad (1)$$

where

n_{HC} – number of moles of the macrocycle formed during synthesis, mmol;
N_{mon} – number of monomeric units in the macrocycle;
$n_{st.mon}$ – total number of moles of starting monomers, mmol;
m_{RM} – total mass of reactant mixture, including internal standard, mg;
m_S – mass of the aged crude mixture used in the sample preparation, mg;
V_S – volume of solvent mixture used in the sample preparation, mL;
S_{HC} – peak area of the macrocycle, mAU·s;
S_{IS} – peak area of internal standard, mAU·s;
m_{IS} – mass of internal standard, mg;
P_{IS} – purity of internal standard, %;
k_{HC} – slope of the macrocycle calibration curve;
k_{IS} – slope of the internal standard calibration curve, mAU·s·mL/mg;
M_{HC} – molar weight of the macrocycle, g/mol.

Powder X-ray Diffraction Analysis (PXRD)

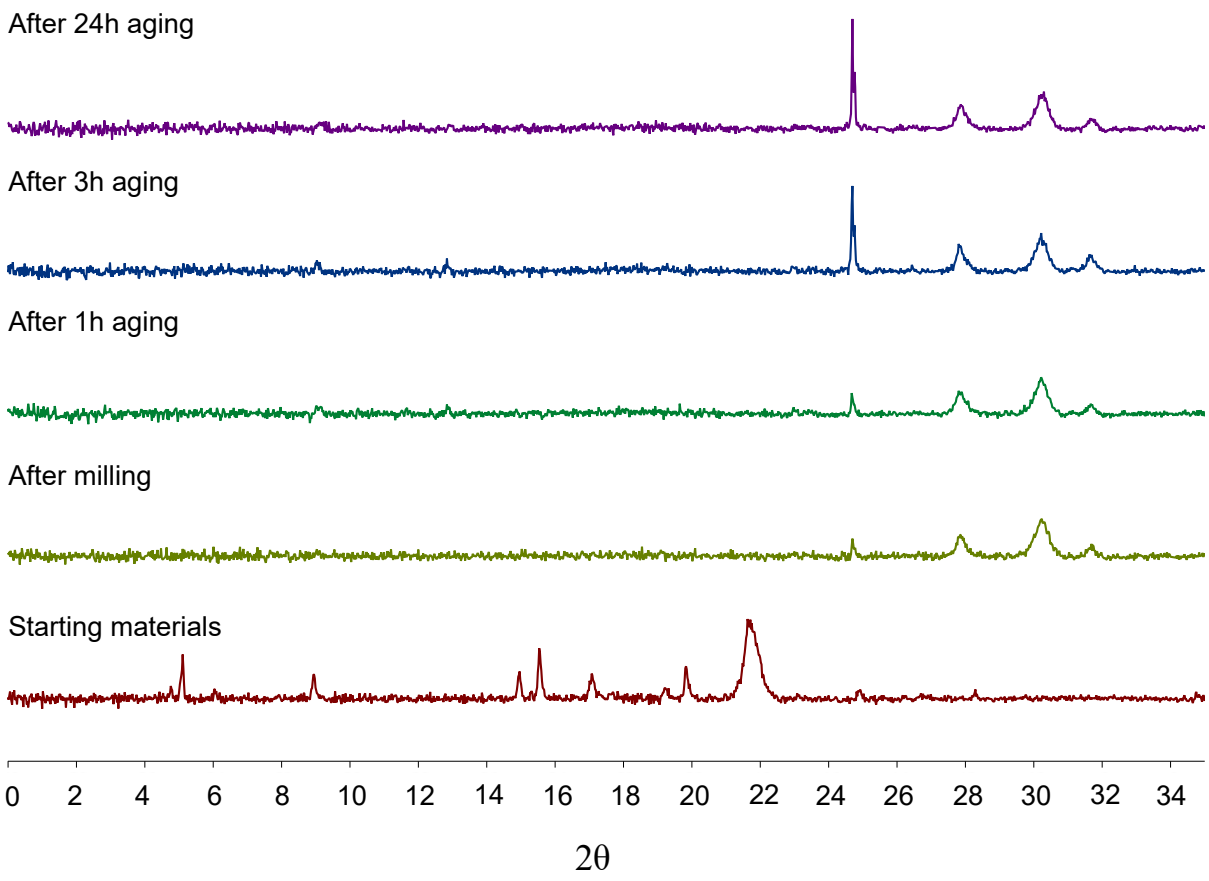


Figure S1. PXRD pattern of starting materials (D-biotin, (*R*,*R*)-*N*,*N*'-cyclohexa-1,2-diylurea, paraformaldehyde, $HClO_4$), crude mixture after milling (1 h milling at 30 Hz) and after aging (1 h milling at 30 Hz + 1 h aging at 60 °C, 1 h milling at 30 Hz + 3 h aging at 60 °C, 1 h milling at 30 Hz + 24 h aging at 60 °C).

PXRD unambiguously confirmed that samples are amorphous in nature. XRD pattern of starting materials exhibits some crystallinity due to the fact that D-biotin, (*R*,*R*)-*N*,*N*'-cyclohexa-1,2-diylurea, paraformaldehyde are solid compounds containing some crystalline phases. Upon milling and aging no formation of crystalline phases was observed in the samples. Peaks at diffraction angles 2θ = 30°, 2θ = 33°, 2θ = 35° and 2θ = 37° correspond to inorganic residue.

Fourier-Transform Infrared Spectroscopy (FTIR)

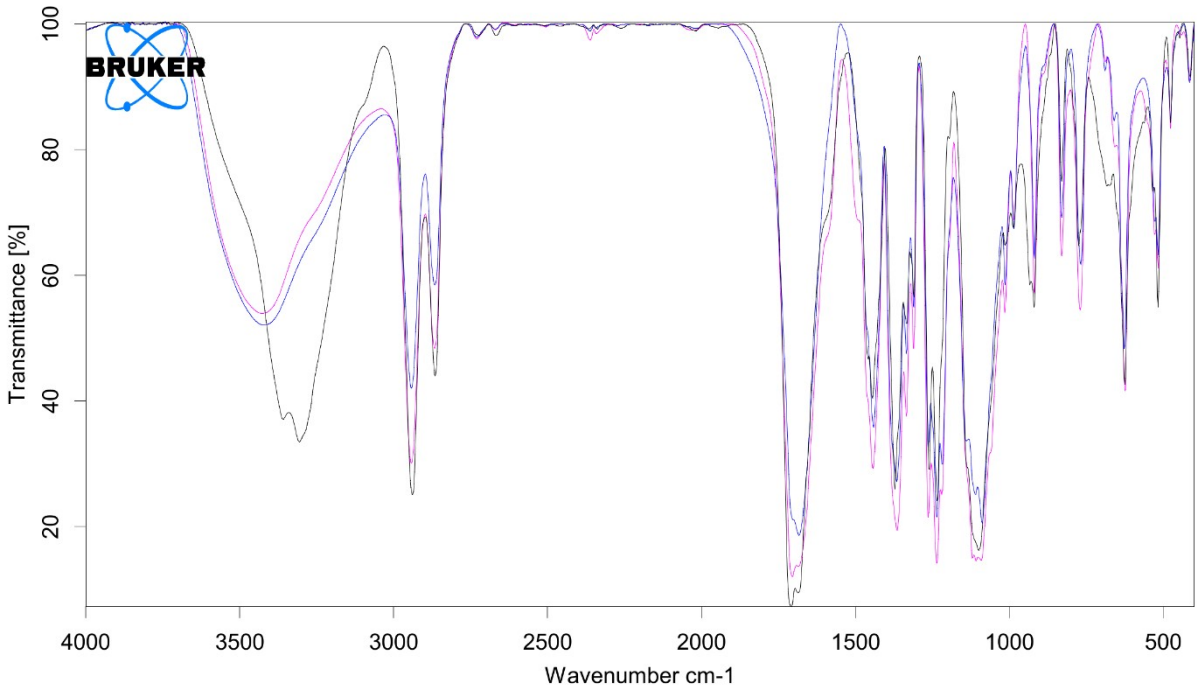


Figure S2. Overlaid FTIR spectra of starting materials (black), crude mixture after 1 h milling (blue) and crude mixture after 1 h milling + 24 h aging (magenta).

Characteristics of the IR spectra of the samples from reaction mixtures

IR (KBr pellet), ν (cm^{-1}):

- **3432–3305** – OH and NH stretching
- **2942–2939** – C-H stretching (cyclohexyl methylene groups)
- **2866–2865** – C-H stretching (cyclohexyl methine groups)
- **1710–1705** – C=O stretching
- **1445–1436** – C-H bending (cyclohexyl methylene groups)
- **1358** – NH bending, C-N bending and stretching
- **1334–1332** – C-H bending (cyclohexyl methine groups)
- **1236–1231** – C-N and C-C stretching
- **1130–1013** – C-C stretching, C-H bending, C-N stretching, and NH bending

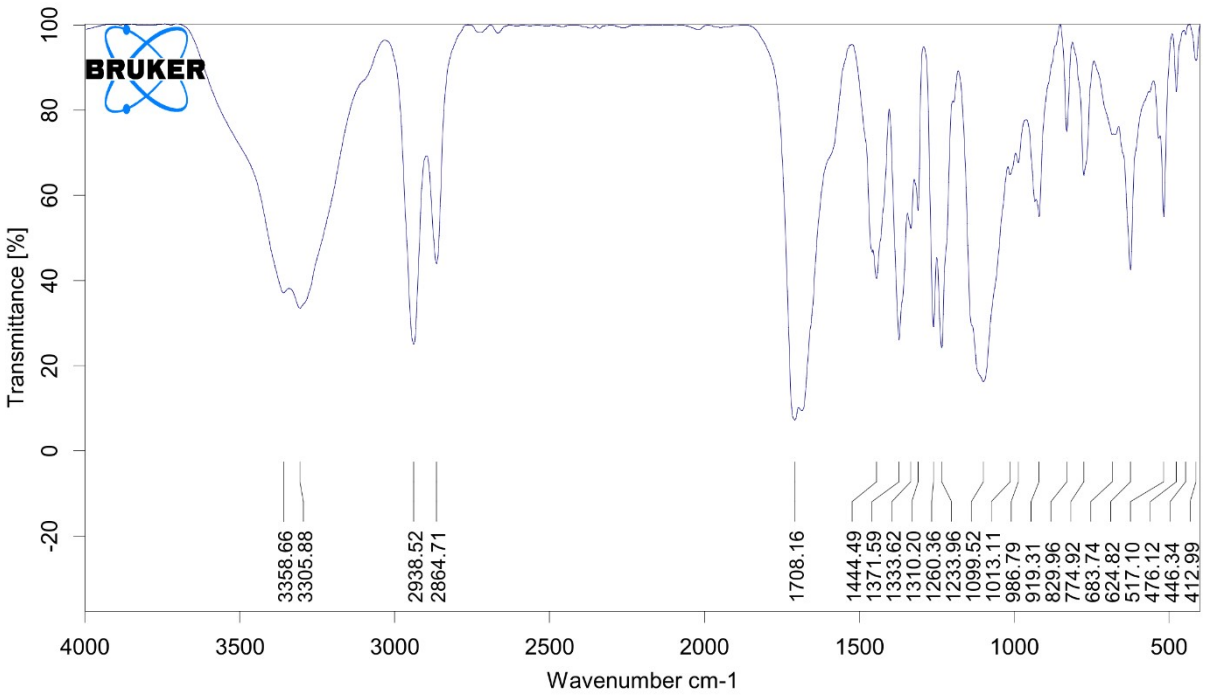


Figure S3. FTIR spectrum of the starting materials (D-biotin, (*R*,*R*)-*N*,*N*'-cyclohexa-1,2-diylurea, paraformaldehyde, $HClO_4$).

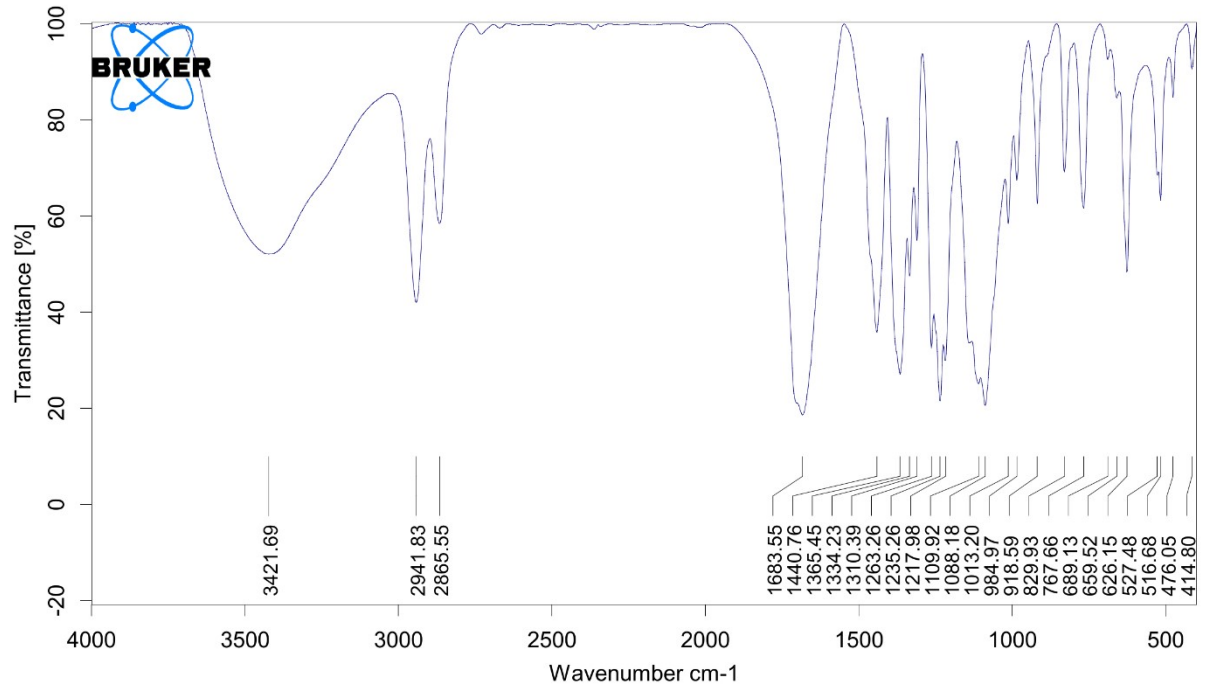


Figure S4. FTIR spectrum of the crude mixture after 1 h milling at 30 Hz.

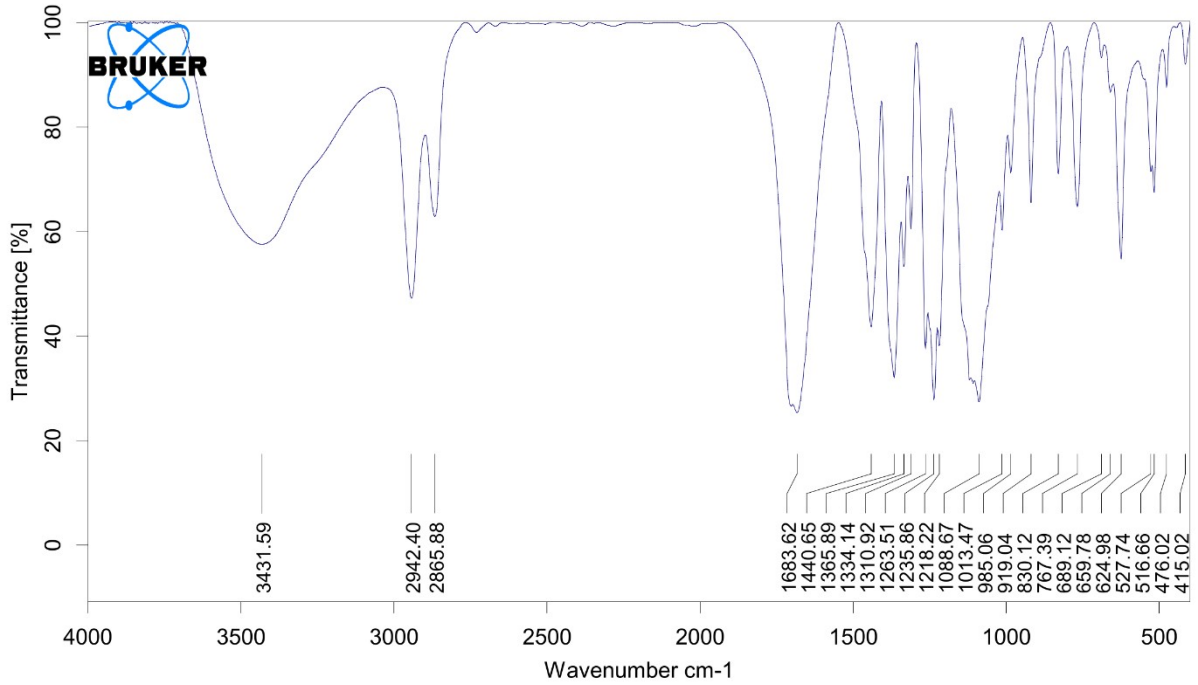


Figure S5. FTIR spectrum of the crude mixture after 1 h milling at 30 Hz + 3 h aging at 60 °C.

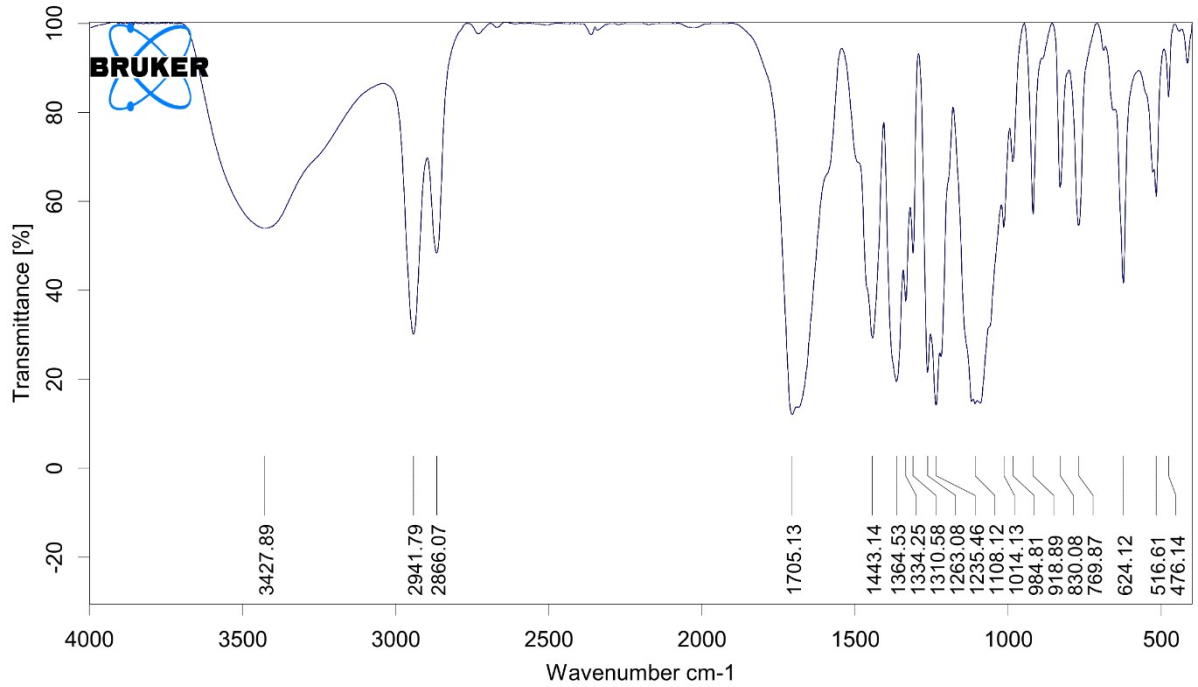


Figure S6. FTIR spectrum of the crude mixture after 1 h milling at 30 Hz + 24 h aging at 60 °C.

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

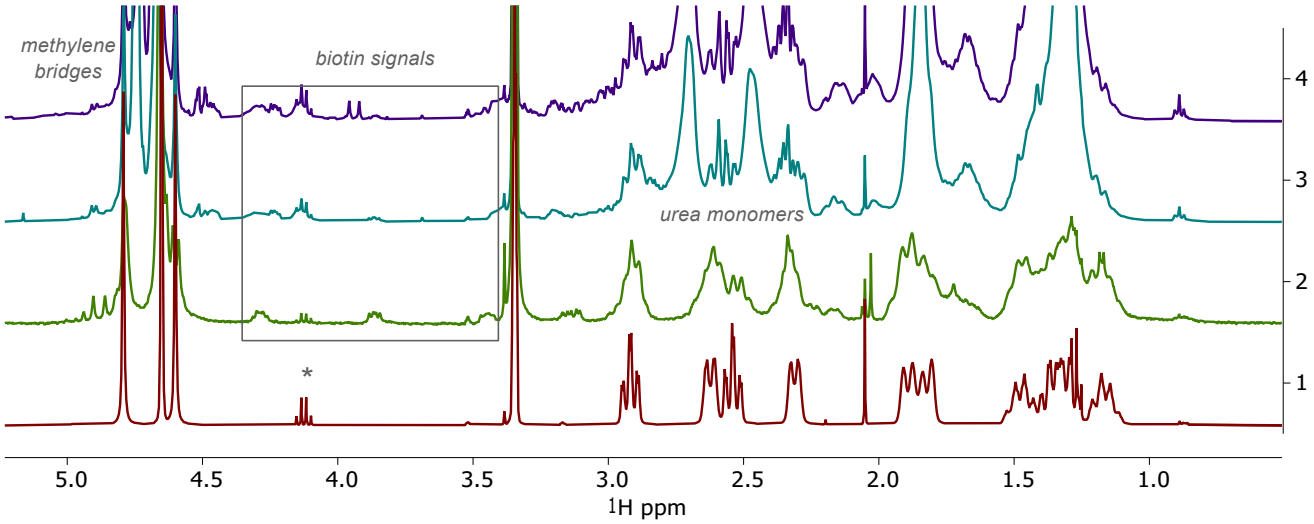


Figure S7. Stacked ^{1}H NMR spectra of pure macrocycles – (*R*,*R*)-cycHC[8] (1) and (–)-mixHC[8] (2), and crude mixture after milling (3) and after aging (4). * - peaks belonging to ethyl acetate.

Detailed assaignment of signals of cycHC[8][1] and mixHC[8][2] is described in our previously published works.

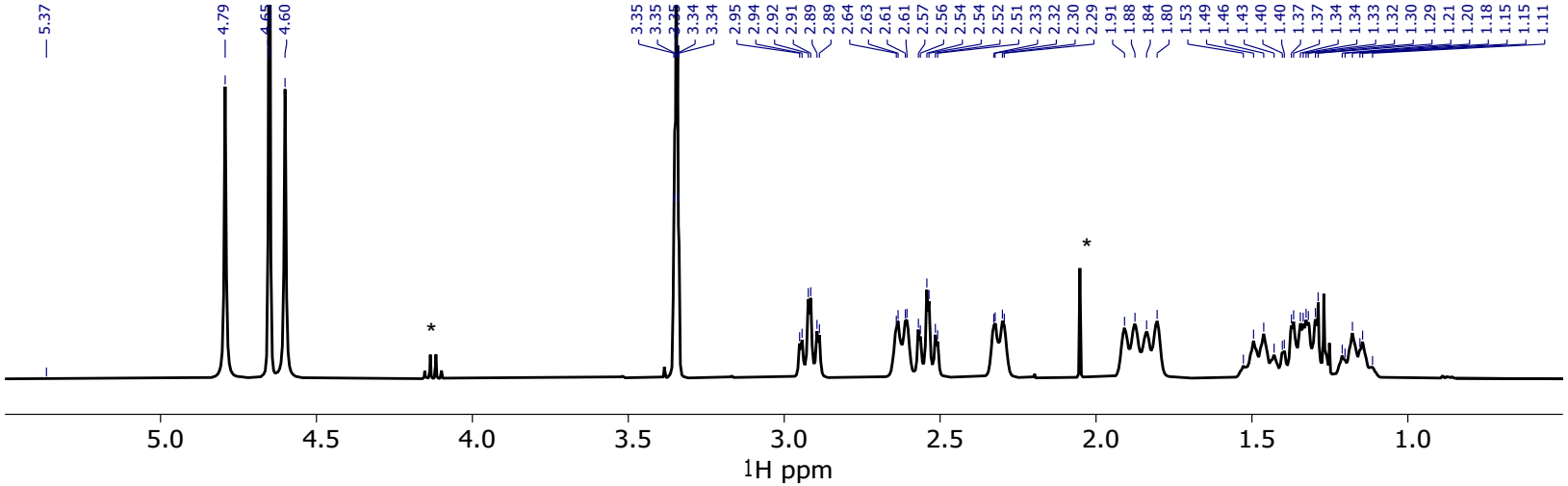


Figure S8. ^{1}H NMR spectrum of (*R,R*)-cycHC[8] in $CDCl_3$:MeOD (1:1) mixture. * is EtOAc

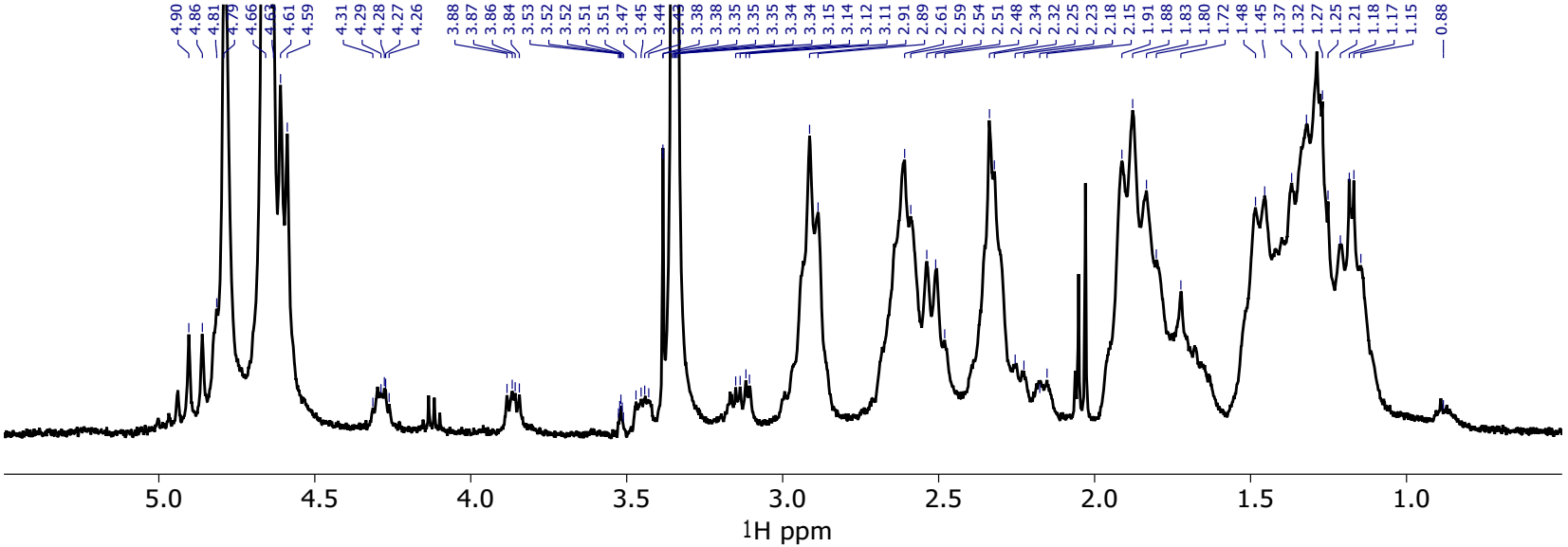


Figure S9. ^{1}H NMR spectrum of (–)-mixHC[8] in $CDCl_3$:MeOD (1:1) mixture.

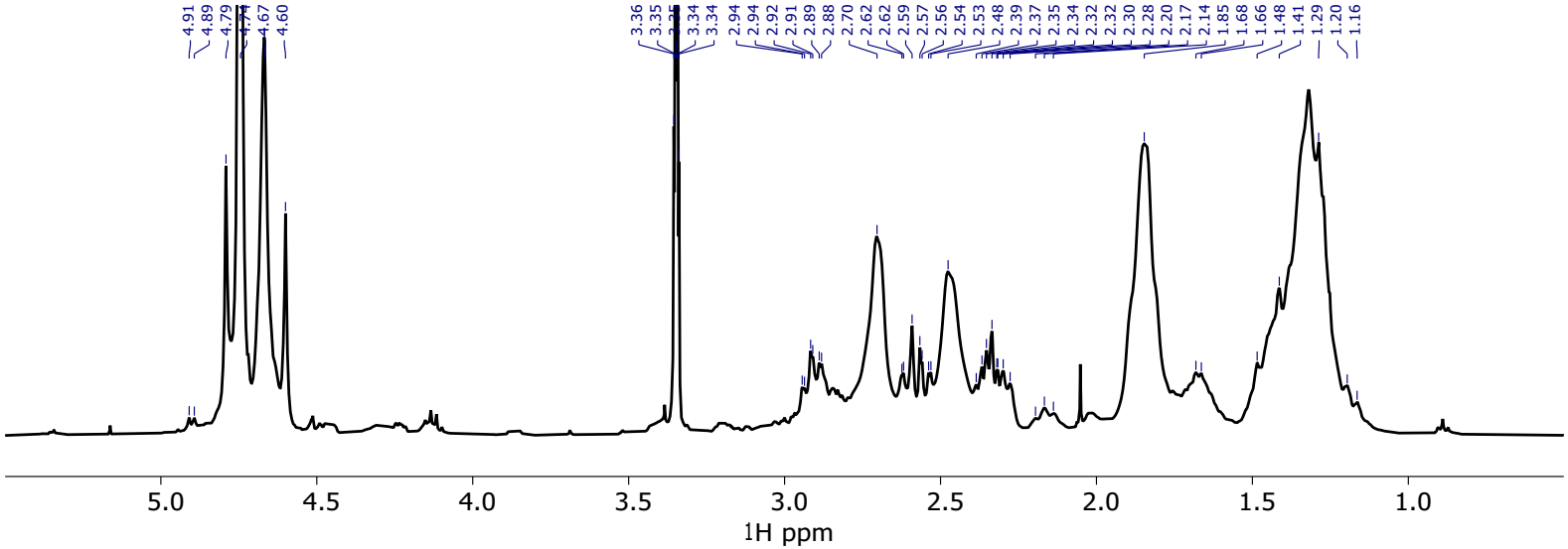


Figure S10. ^{1}H NMR spectrum of mixHC[8] crude mixture after 1 h milling in $CDCl_3$:MeOD (1:1) mixture.

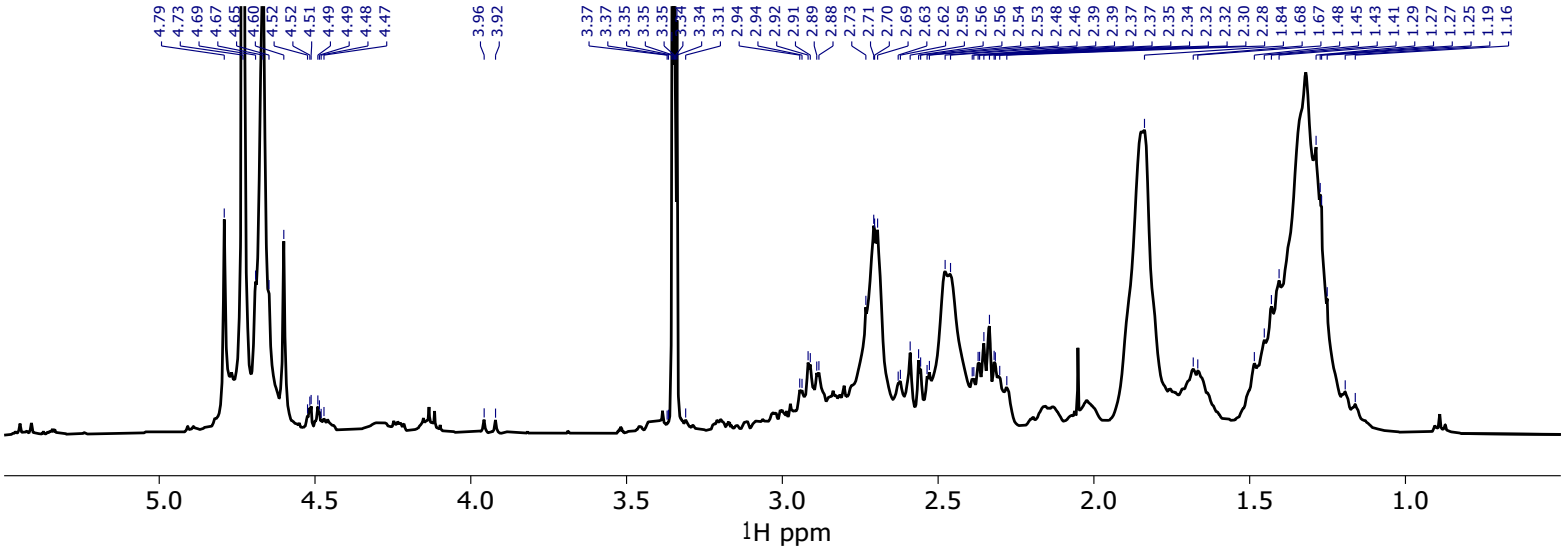


Figure S11. ^{1}H NMR spectrum of mixHC[8] crude mixture after 1 h milling + 24 h aging in $CDCl_3$:MeOD (1:1) mixture.

Mass Spectrometry (MS)

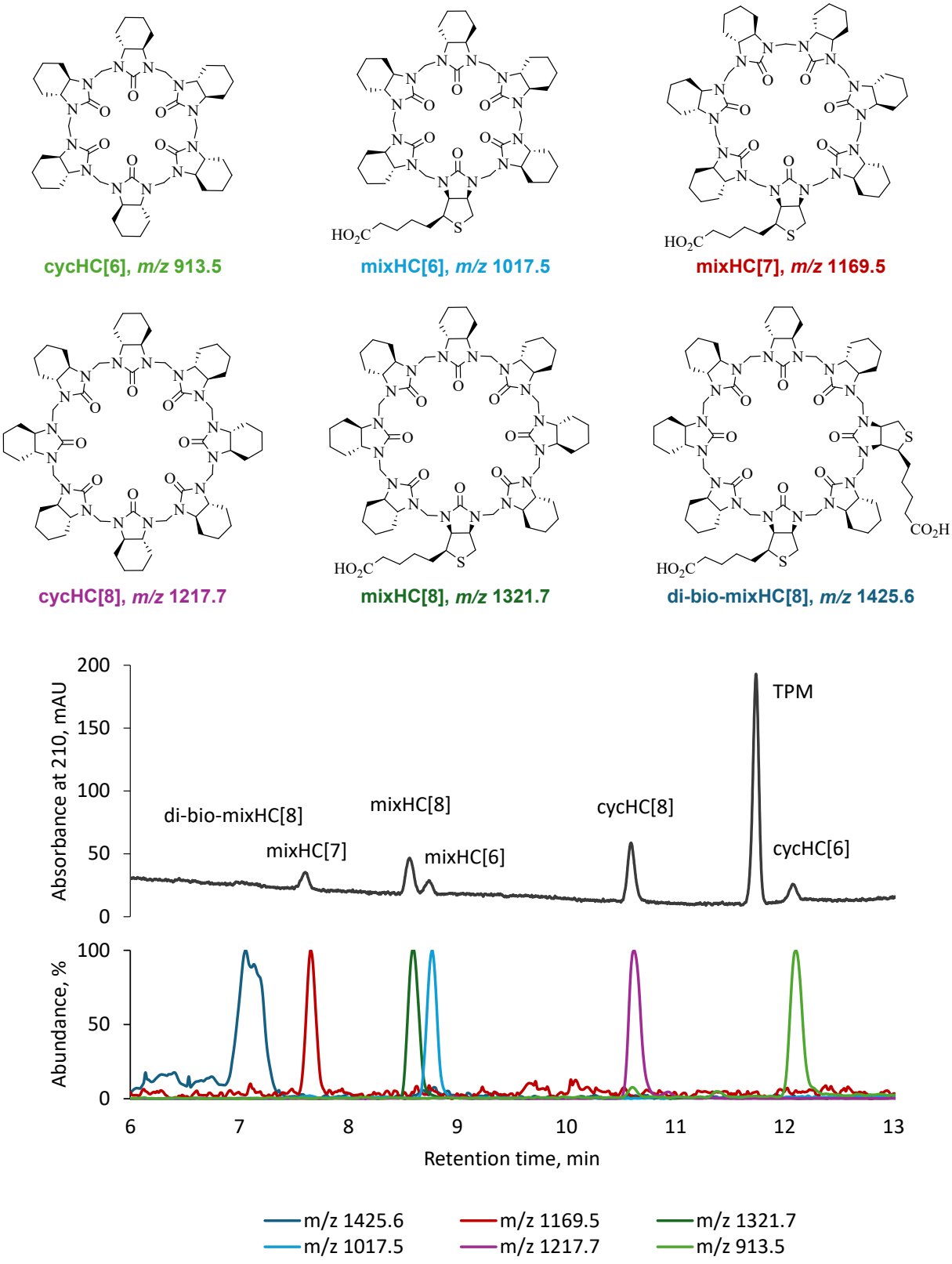


Figure S12. Macrocycles detected by HPLC-UV-MS analysis in the crude mixtures (upper chromatogram: UV detection at 210 nm, lower: extracted ion chromatograms corresponding to

protonated macrocycles, $[M+H]^+$, normalized to the largest peak). Note: biotin units in di-bio-mixHC[8] can be incorporated in different positions.

Table S1. Theoretical and experimental *m/z* values for different macrocyclic species

Macrocycle	Ion	Formula	*m/z* theor	*m/z* exp[a]
cycHC[6]	$[M+H]^+$	$C_{48}H_{73}N_{12}O_6^+$	913.58	913.50
	$[M+Na]^+$	$C_{48}H_{72}N_{12}O_6Na^+$	935.56	935.48
mixHC[6]	$[M+H]^+$	$C_{51}H_{77}N_{12}O_8S^+$	1017.57	1017.45
	$[M+Na]^+$	$C_{51}H_{76}N_{12}O_8SNa^+$	1039.55	1039.40
mixHC[7]	$[M+H]^+$	$C_{59}H_{89}N_{14}O_9S^+$	1169.67	1169.53
	$[M+Na]^+$	$C_{59}H_{88}N_{14}O_9SNa^+$	1191.65	1191.50
cycHC[8]	$[M+H]^+$	$C_{64}H_{97}N_{16}O_8^+$	1217.77	1217.70
	$[M+Na]^+$	$C_{64}H_{96}N_{16}O_8Na^+$	1239.75	1239.69
mixHC[8]	$[M+H]^+$	$C_{67}H_{101}N_{16}O_{10}S^+$	1321.76	1321.67
	$[M+Na]^+$	$C_{67}H_{100}N_{16}O_{10}SNa^+$	1343.74	1343.60
di-bio-mixHC[8]	$[M+H]^+$	$C_{70}H_{105}N_{16}O_{12}S_2^+$	1425.75	1425.64
	$[M+Na]^+$	$C_{70}H_{104}N_{16}O_{12}S_2Na^+$	1447.74	1447.64

[a] Experimental *m/z* values were obtained by (+)ESI-MS on a single quadrupole detector with mass accuracy ±0.13 Da.

High-Perfromance Liquid Chromatography (HPLC)

Stability

Table S2. Stability of sample solution in a solvent mixture with different alcohols

Time, h	$CHCl_3$:MeOH (1:1)				$CHCl_3$:iPrOH (1:1)		
	$c_{mixHC[8]}$, µg/mL	$c_{mixHC[8]\ Me\ ester}$, µg/mL	$c_{cycHC[8]}$, µg/mL	c_{TPM}, µg/mL	$c_{mixHC[8]}$, µg/mL	$c_{cycHC[8]}$, µg/mL	c_{TPM}, µg/mL
0	160	0	165	25	162	171	29
1.4	153	11	166	25	159	172	29
2.6	149	16	169	24	160	169	30
3.8	141	25	167	25	159	171	30
7.1	124	36	168	25	162	169	30
12.3	105	51	166	24	160	169	30
24	87	68	167	24	163	172	29

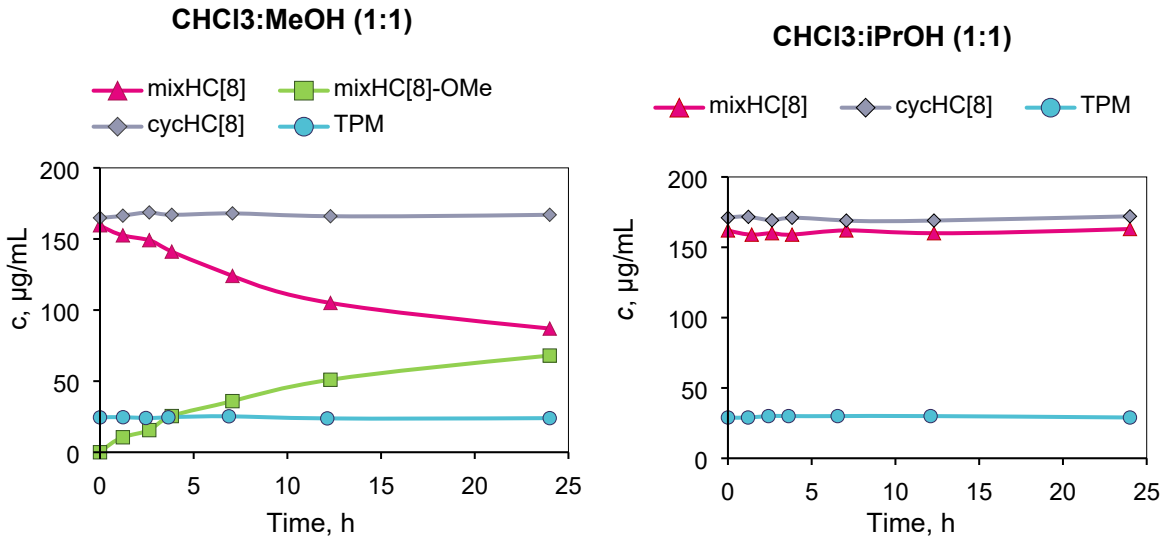


Figure S13. Stability of the crude mixture sample in solvent mixtures with different alcohols. The formation of mixHC[8] methyl ester was confirmed by (+)ESI-MS: *m/z* calcd. for $C_{68}H_{103}N_{16}O_{10}S^+$ $[M+H]^+$ 1335.8, found *m/z* 1335.6.

Calibration, linearity, LoQ, LoD

Calibration curves were obtained for a series of solutions prepared in isopropanol : chloroform 1:1 (v/v) mixture and containing TPM and analytes at the following concentrations:

TPM (*P*=99%): 0.1 mg/mL, 0.09 mg/mL, 0.08 mg/mL, 0.07 mg/mL, 0.06 mg/mL, 0.05 mg/mL, 0.04 mg/mL, 0.03 mg/mL, 0.02 mg/mL, 0.01 mg/mL, 0.0025 mg/mL;

mixHC[8] (P_{NMR}=90%), ***cycHC[8]*** (P_{NMR}=96%), ***cycHC[6]*** (P_{NMR}=98%):

0.9 mg/mL, 0.8 mg/mL, 0.7 mg/mL, 0.6 mg/mL, 0.5 mg/mL, 0.4 mg/mL, 0.3 mg/mL, 0.2 mg/mL, 0.1 mg/mL, 0.025 mg/mL.

The collected data was used to evaluate method linearity, LoQ and LoD.

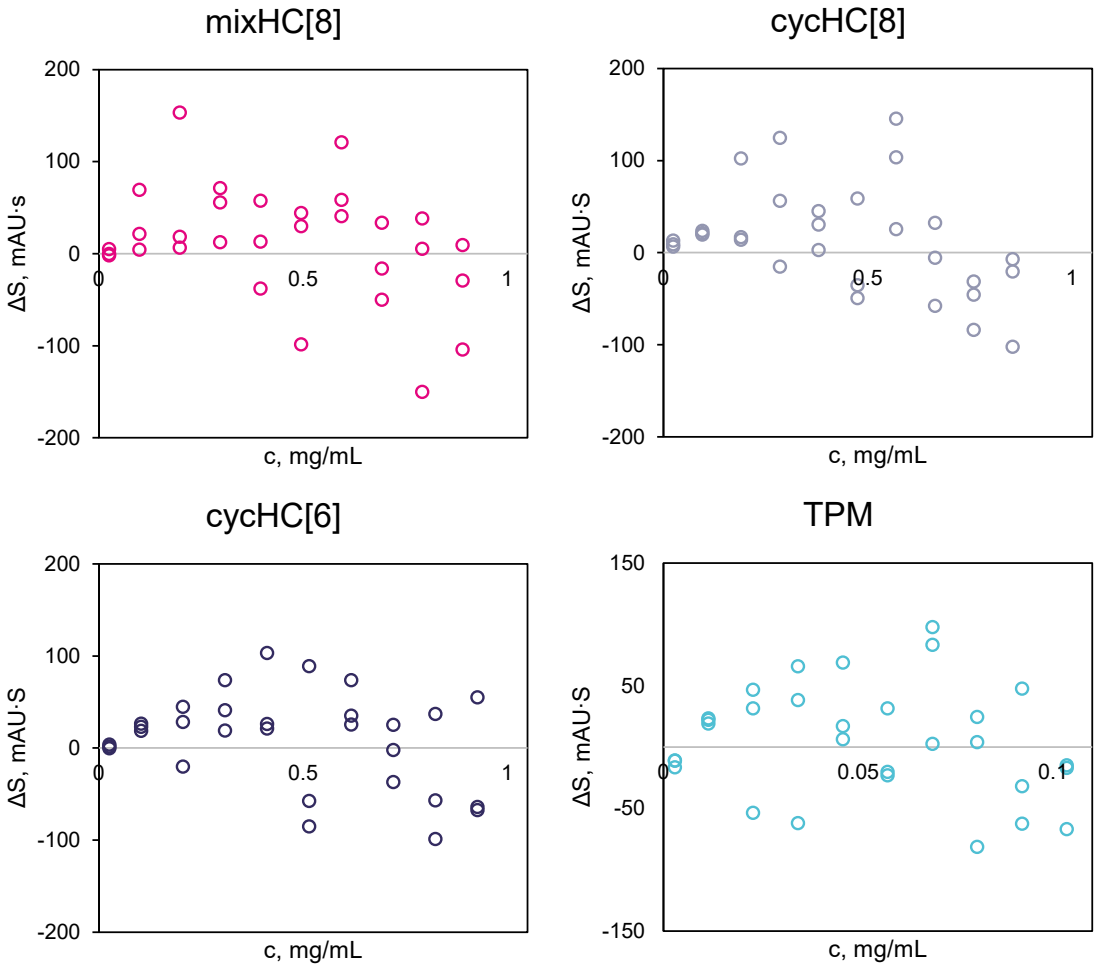


Figure S14. Plots of residuals used in evaluation of linearity (the residuals express the difference between the experimentally observed peak areas, and peak areas predicted using the linear regression model $\Delta S = S_{exp} - S_{calc}$).

Table S3. Method linearity, LoQ and LoD

Compound	Slope of calibration curve, *k*	Correlation coefficient, R^2	LoQ, µg/mL	LoD, µg/mL
TPM	21800±100	0.9989	2.5	1
mixHC[8]	2990±20	0.9985	25	10
cycHC[8]	3080±20	0.9986	25	10
cycHC[6]	2660±20	0.9988	25	10

Internal standard: inertness and distribution during ball milling

The internal standard intertness was assessed based on its recovery (R, %) after milling, calculated via formula (2):

$$R_i = \frac{\omega_{TPM,\ exp}}{\omega_{TPM,\ theor}} \cdot 100 \qquad (2)$$

where R_i – the internal standard recovery in a single milling experiment i, %; $\omega_{TPM,exp}$ – the percentage of the internal standard in the reaction mixture according to the results of the analysis, % $\omega_{TPM,theor}$ – theoretical percentage of the internal standard in the reaction mixture, calculated based on the weighed amount, %.

Table S4. TPM recovery upon milling under neat grinding and LAG conditions.

Entry	Liquid additive	Milling time, h	$\omega_{TPM,\ theor}$, %	$\omega_{TPM,\ exp}$, %	R_i, %	R_{mean}, %
1[a]	HPF_6	1	5.38[b]	5.29±0.03[b]	98±1	98.7±0.5
2[a]			5.23[b]	5.18±0.07[b]	99±1	
3	–	1	3.25	3.20±0.03	99±1	98.2±0.4
4			3.22	3.16±0.05	98±2	
5	HPF_6	1	2.49	2.37±0.03	95±1	96±4
6			2.16	2.18±0.05	101±2	
7			2.17	2.03±0.04	94±2	
8		2	2.49	2.34±0.02	94±1	95±3
9			2.16	2.13±0.06	99±3	
10			2.17	2.01±0.01	92.5±0.3	
11	H_2O	1	2.48	2.24±0.03	90±1	112±20
12			2.46	3.2±0.2	129±8	
13			2.46	2.83±0.07	115±3	
14	DMSO	1	2.56	2.54±0.02	99±1	98±2
15			2.48	2.38±0.05	96±2	

[a] Instead of replicate sampling, the entire crude mixture was analyzed. [b] Mass of internal standard, mg.

The crude mixtures from entries 1 and 2 were analyzed via quantitative transfer of the whole mixture into a 100 mL measuring flask and dissolving it in the solvent mixture. The rest of the analysis was carried out according to the procedure.

Distribution of internal standard

A. Concentration of internal standard

Table S5. Effect of TPM amount on the distribution of reaction mixture components

Entry	Solid starting materials, mg	η, µL/mg	c_{TPM}, %	*RSD* of peak area, %		
				TPM	mixHC[8]	cycHC[8]
1	301	0.16	1.5	2.4	1.3	4.7
2	299	0.17	1.2	3.0	3.4	1.0
3[a]	307	0.17	1.4	1.8	2.6	2.2
4	304	0.18	1.3	1.4	1.7	0.4
5	286	0.17	2.8	0.8	0.7	2.4
6	303	0.18	2.7	0.8	4.5	2.1
7	290	0.18	4.0	0.8	2.7	2.7
8	306	0.18	3.7	4.7	3.2	4.2
9	310	0.17	2.5	0.7	0.8	0.2
10	311	0.18	2.5	0.7	0.9	1.4
11[b]	297	0.18	2.5	1.9	2.4	2.8
12	309	0.19	2.6	0.8	0.5	1.5
13	309	0.19	2.6	2.0	3.1	2.5
14	307	0.19	2.5	1.3	0.6	0.9
15	303	0.18	2.7	1.8	1.7	0.9
16	302	0.18	2.4	3.4	3.0	2.1
17	292	0.14	2.8	0.2	1.6	0.6
18	293	0.13	3.1	0.8	0.7	1.7
19	307	0.19	2.8	1.5	1.3	2.6
20	307	0.19	2.2	1.4	0.9	1.9
21	310	0.18	2.4	0.7	2.5	2.5
22	307	0.19	2.5	1.4	0.7	1.3
23	301	0.20	2.5	0.2	2.0	0.5
24	309	0.19	2.6	0.6	1.2	1.2
25	306	0.19	2.2	2.2	3.5	1.5
26	308	0.19	2.2	1.3	1.0	1.6
27	308	0.19	2.7	2.2	3.5	2.6
28	308	0.19	2.7	0.1	0.3	2.0
29	307	0.19	2.4	1.2	0.6	2.0
30	307	0.19	2.3	2.5	0.6	0.2
31	308	0.19	2.4	3.1	0.5	0.8

General reaction conditions: D-biotin (1 equiv.), (*R,R*)-*N,N'*-cyclohexa-1,2-diylurea (7 equiv.), paraformaldehyde (8 equiv.), acid (2 equiv.) and salt additive (1 equiv.) were milled at 30 Hz for 60 min and aged at 60 °C for 24 h. The crude mixtures after aging were analyzed in triplicate (n=3), except [a] n=9, [b] n=6.

B. LAG effect

Table S6. Effects of LAG additive and jar loading on the distribution of reaction mixture components

Entry	Solids[a], mg	η, µL/mg	c_{TPM}, %	*RSD* of peak area, %		
				TPM	mixHC[8]	cycHC[8]
1	283	0.5	1.8	2.3	3.0	4.3
2	327	0.16	3.4	0.5	2.0	1.3
3	325	1.15	1.7	5.9	3.3	1.2
4	389	0.64	1.7	5.0	4.0	3.1
5	387	0.64	1.5	6.1	4.1	4.3
6	278	0.41	2.3	1.5	0.7	2.0
7	284	0.85	1.7	10.4	8.3	19.5
8	279	0.15	3.5	0.8	1.2	1.3
9	280	0.41	2.0	3.2	1.1	4.8
10	352	0.45	1.7	1.4	3.0	1.5
11	384	0.45	1.8	1.1	1.3	2.3
12	288	0.61	1.9	3.1	33.0	18.1
13	337	0.29	2.2	3.8	3.3	2.3
14	295	0.26	2.6	1.3	0.7	3.1
15	380	0.56	1.4	5.5	10.3	5.2
16	344	0.68	1.4	6.5	8.1	7.3
17	330	0.54	2.3	1.4	3.2	2.2
18	369	0.84	1.3	8.2	16.3	16.9
19	282	0.41	2.4	1.7	0.9	2.0

General reaction conditions: D-biotin (1 equiv.), (*R,R*)-*N,N'*-cyclohexa-1,2-diylurea (7 equiv.), paraformaldehyde (8 equiv.), KPF_6 (0–3 equiv.), 55% aq. HPF_6 (0.1–8 equiv.) and water (LAG additive, total η=0.2–0.85 µl/mg) were milled for 60 min and aged at 60 °C for 24 h. [a] Solid starting materials and internal standard. Each reaction was analyzed in triplicate.

C. Mass of solids

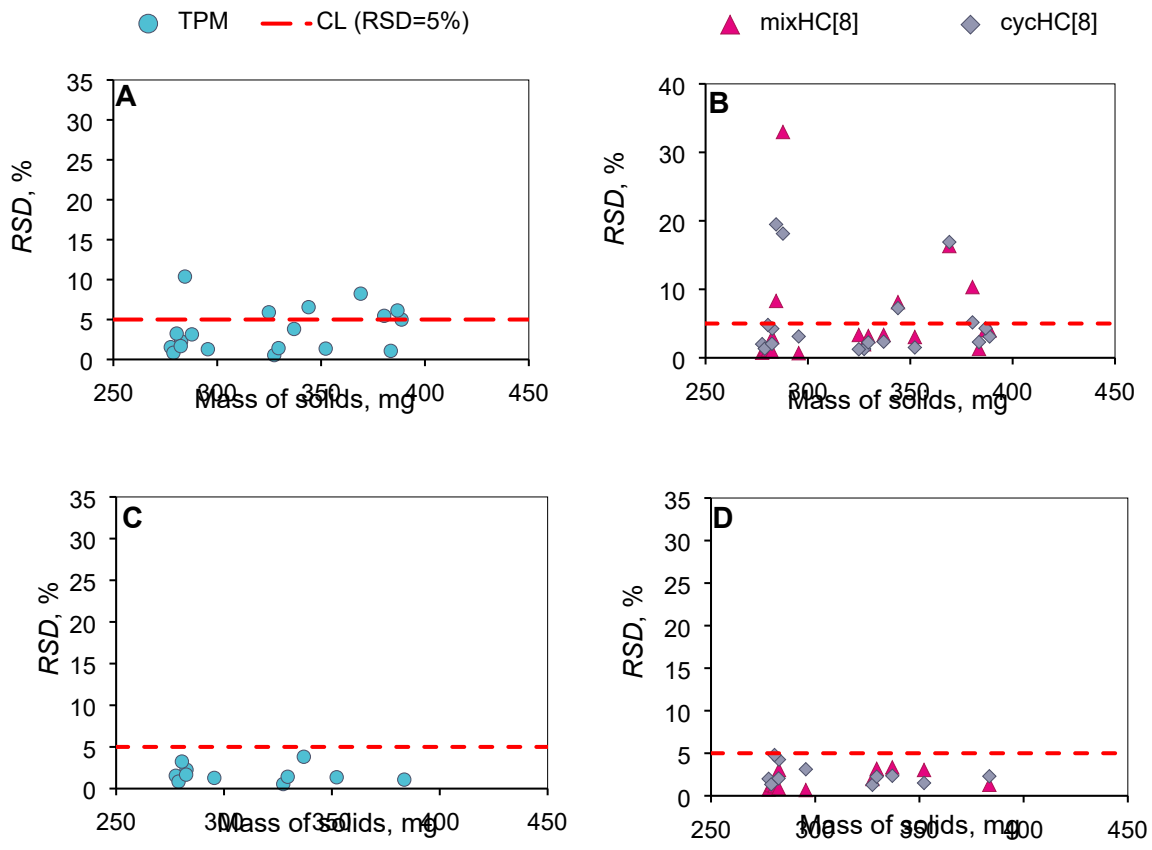


Figure S15. Distribution of internal standard and products upon milling depending on the mass of solids under η=0.15–1.15 μL/mg (A, B) and η<0.56 μL/mg (C, D) LAG conditons.

D. Milling duration

Table S7. Effect of milling duration on the distribution of reaction mixture components

Entry	Solid starting materials, mg	η, µL/mg	c_{TPM}, %	Milling time, min	*RSD* of peak area, %		
					TPM	mixHC[8]	cycHC[8]
1	310	0.18	2.4	60	0.7	2.5	2.5
2	307	0.19	2.5	60	1.4	0.7	1.3
3	301	0.20	2.5	60	0.2	2.0	0.5
4	307	0.19	2.7	5	1.8	1.5	4.1
5	308	0.19	2.2	10	1.7	1.7	1.1
6	308	0.19	2.5	20	2.5	3.1	1.2
7	310	0.19	2.5	30	0.3	0.9	1.1
8	308	0.19	2.4	45	0.4	1.2	4.2
9	309	0.19	2.6	60	0.6	1.2	1.2
10	308	0.19	2.5	5	1.3	3.9	3.9
11	307	0.19	2.4	10	0.8	1.6	1.7
12	307	0.19	2.4	20	1.1	1.0	1.3
13	308	0.19	2.1	30	2.9	1.6	4.7
14	309	0.19	2.6	45	0.9	1.9	2.1
15	306	0.19	2.2	60	2.2	3.5	1.5
16	307	0.19	2.8	90	2.4	2.6	2.2
17	309	0.19	2.6	90	1.5	1.3	1.9
18	309	0.19	2.2	30	1.5	2.5	3.2
19	307	0.19	2.3	45	0.8	1.8	2.3
20	309	0.19	2.4	5	3.0	4.1	3.0
21	307	0.19	2.5	10	1.5	2.8	2.1
22	307	0.19	2.3	20	1.9	1.9	1.4
23	308	0.19	2.6	30	3.6	2.8	1.3
24	308	0.19	2.5	45	3.8	2.6	2.4
25	307	0.19	2.5	60	1.3	0.7	0.6

Each reaction was analyzed in triplicate.

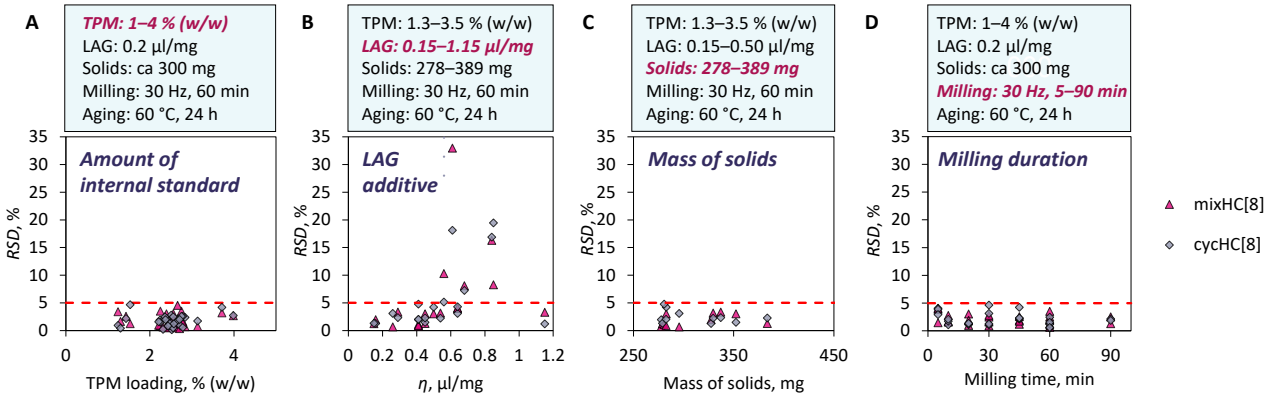


Figure S16. Distribution of products during milling depending on different paramters

Distribution of products

Table S8. Distribution of the products and internal standard under different reaction conditions.

Entry	Monomer ratio[a]	Template	Milling time, min	Aging	Solids, mg	η, μL/mg	HPLC yield, %		*RSD* of peak area, %		
							mixHC[8]	cycHC[8]	mixHC[8]	cycHC[8]	TPM
1	1.0 : 11.0	$HClO_4$ (1.9 equiv.)	36	35°C, 24 h	310.7	0.11	9.5±0.3	17.9±0.3	1.4	2.5	2.5
2	1.0 : 11.0	$HClO_4$ (1.9 equiv.)	36	35°C, 24 h	306.6	0.11	7.9±0.1	16.5±0.1	2.3	0.7	1.3
3	1.0 : 11.1	$HClO_4$ (4.0 equiv.)	37	59°C, 24 h	309.6	0.23	8.4±0.3	33±1	4.7	2.9	1.3
4	1.0 : 11.1	$HClO_4$ (4.0 equiv.)	37	59°C, 24 h	309.8	0.23	6.9±0.3	36±1	7.7	3.1	3.9
5	1.0 : 6.6	$HClO_4$ (0.5 equiv.)	60	75°C, 24 h	299.9	0.03	2.0±0.1	3.6±0.4	3.6	11.9	2.2
6	1.0 : 15.0	$HClO_4$ (4.0 equiv.)	10	35°C, 24 h	301.2	0.23	9±1	50.6±0.3	9.1	1.4	1.6
7	1.0 : 8.5	$HClO_4$ (2.7 equiv.)	10	49°C, 24 h	302.5	0.16	16.5±0.2	43.5±0.2	2.3	1.8	1.4
8	1.0 : 8.5	$HClO_4$ (2.7 equiv.)	10	49°C, 24 h	302.4	0.16	14±1	40±2	2.2	1.2	3.0
9	1.0 : 15.0	$HClO_4$ (0.5 equiv.)	60	75°C, 24 h	301.1	0.18	7±1	16.5±0.3	7.2	2.2	2.4
10	1.0 : 6.6	$HClO_4$ (0.5 equiv.)	60	75°C, 24 h	299.1	0.03	4.6±0.1	5.5±0.2	1.6	4.2	0.5
11	1.0 : 15.0	$HClO_4$ (3.0 equiv.)	60	45°C, 24 h	299.7	0.18	20±1	72±1	2.6	0.3	1.6
12	1.0 : 7.9	$HClO_4$ (0.5 equiv.)	60	45°C, 24 h	300.3	0.03	2.0±0.2	3.0±0.2	9.3	5.9	1.6
13	1.0 : 5.0	$HClO_4$ (1.8 equiv.)	37	59°C, 24 h	300	0.10	16.4±0.3	24±1	0.9	0.9	1.3
14	1.0 : 5.0	$HClO_4$ (1.8 equiv.)	37	59°C, 24 h	299.9	0.10	17±1	28±1	2.0	3.7	0.7
15	1.0 : 15.0	$HClO_4$ (3.0 equiv.)	13	75°C, 24 h	302.4	0.17	14.6±0.4	50±1	2.5	1.7	0.7
16	1.0 : 8.0	$HClO_4$ (0.5 equiv.)	12	75°C, 24 h	303.3	0.03	5.4±0.1	9.3±0.4	1.5	4.0	1.1
17	1.0 : 15.0	$HClO_4$ (0.5 equiv.)	50	75°C, 24 h	302.1	0.03	4.0±0.1	11.7±0.1	2.6	2.3	2.1
18	1.0 : 5.0	$HClO_4$ (4.0 equiv.)	10	75°C, 24 h	301	0.22	1.6±0.3	1.9±.0.2	14.0	11.1	2.2
19	1.0 : 5.0	$HClO_4$ (4.0 equiv.)	60	35°C, 24 h	300.8	0.22	9.0±0.2	16±1	1.6	5.2*	1.3
20	1.0 : 5.0	$HClO_4$ (4.0 equiv.)	26	35°C, 24 h	301.2	0.22	11±1	16.9±.2	4.8	1.7	1.0
21	1.0 : 15.0	$HClO_4$ (0.5 equiv.)	13	47°C, 24 h	301.4	0.03	2.1±0.2	6±1	9.7	8.9	0.4
22	1.0 : 11.4	$HClO_4$ (1.8 equiv.)	48	59°C, 24 h	301.1	0.10	19.9±0.2	44.3±0.4	1.7	0.9	0.8
23	1.0 : 7.0	HPF_6 (5.0 equiv.)	60	60°C, 24 h	272.5	0.50	30±1	35±1	3.0	4.3	2.3
24	1.0 : 7.0	HPF_6 (0.7 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	313.9	0.16	18.6±0.3	23.3±0.2	2.0	1.3	0.5
25	1.0 : 7.0	HPF_6 (6.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	310.7	1.15	29±1	34±2	3.3	1.2	5.9
26	1.0 : 7.0	HPF_6 (0.5 equiv.), KPF_6 (3.0 equiv.)	60	60°C, 24 h	377.7	0.64	8±1	21±2	4.0	3.1	5.0
27	1.0 : 7.0	HPF_6 (3.1 equiv.), KPF_6 (3.0 equiv.)	60	60°C, 24 h	376.5	0.64	29.6±0.4	35±1	4.1	4.3	6.1
28	1.0 : 7.0	HPF_6 (3.8 equiv.)	60	60°C, 24 h	267.1	0.41	28.6±0.4	29.5±0.2	0.7	2.0	1.5
29	1.0 : 7.0	HPF_6 (8.0 equiv.)	60	60°C, 24 h	273.1	0.85	11.3±0.3	34±3	8.3	19.5	10.4
30	1.0 : 7.0	HPF_6 (1.0 equiv.)	60	60°C, 24 h	266.8	0.15	9.7±0.1	14.5±0.2	1.2	1.3	0.8

Continuation of Table S8

Entry	Monomer ratio[a]	Template	Milling time, min	Aging	Solids, mg	η, µL/mg	HPLC yield, %		*RSD* of peak area, %		
							mixHC[8]	cycHC[8]	mixHC[8]	cycHC[8]	TPM
31	1.0 : 7.0	HPF_6 (3.8 equiv.)	60	60°C, 24 h	270.8	0.41	25±1	47±1	1.1	4.8	3.2
32	1.0 : 7.0	HPF_6 (4.5 equiv.), KPF_6 (2.1 equiv.)	60	60°C, 24 h	341.9	0.45	21.7±0.1	48.7±0.3	1.7	1.5	1.4
33	1.0 : 7.0	HPF_6 (4.0 equiv.), KPF_6 (3.0 equiv.)	60	60°C, 24 h	372.1	0.45	25.2±0.2	43±1	1.3	2.3	1.1
34	1.0 : 7.0	HPF_6 (6.1 equiv.), KPF_6 (0.1 equiv.)	60	60°C, 24 h	276.3	0.61	10±4	33±7	33.0	18.1	3.1
35	1.0 : 7.0	HPF_6 (2.7 equiv.), KPF_6 (1.7 equiv.)	60	60°C, 24 h	327.9	0.29	22.3±0.1	51±1	3.3	2.3	3.8
36	1.0 : 7.0	HPF_6 (2.6 equiv.), KPF_6 (0.3 equiv.)	60	60°C, 24 h	284.2	0.26	24.7±0.2	33±1	0.7	3.1	1.3
37	1.0 : 7.0	HPF_6 (5.6 equiv.), KPF_6 (3.0 equiv.)	60	60°C, 24 h	370.5	0.56	25±1	39±1	10.3	5.2	5.5
38	1.0 : 7.0	HPF_6 (6.8 equiv.), KPF_6 (1.8 equiv.)	60	60°C, 24 h	333.7	0.68	11.1±0.4	46±2	8.1	7.3	6.5
39	1.0 : 7.0	HPF_6 (4.9 equiv.), KPF_6 (1.2 equiv.)	60	60°C, 24 h	315.6	0.54	21.2±0.4	50±1	3.2	2.2	1.4
40	1.0 : 7.0	HPF_6 (8.0 equiv.), KPF_6 (2.6 equiv.)	60	60°C, 24 h	358.3	0.84	20±2	37±3	16.3	16.9	8.2
41	1.0 : 7.0	HPF_6 (3.8 equiv.)	60	60°C, 24 h	270.9	0.41	29±1	38.3±0.3	0.9	2.0	1.7
42	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	–	305.6	0.20	14.8±0.2	20.7±1	0.8	4.1	1.8
43	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	5	–	308.8	0.19	1.8±0.3	4±3	15.0	61.3	0.4
44	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	10	–	307.2	0.20	3.4±0.2	11±1	3.8	4.3	2.1
45	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	20	–	306.9	0.20	7.7±0.3	18.1±0.1	3.2	2.7	2.8
46	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	30	–	307.5	0.20	6.6±0.2	10±1	3.8	4.4	2.5
47	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	45	–	307.7	0.19	11.0±0.3	17±1	1.1	1.5	2.0
48	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	–	307.1	0.20	11.4±0.3	15±1	5.4b	1.9	2.5
49	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	–	303.8	0.18	9.3±0.2	16.6±0.1	0.8	1.2	1.2
50	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	314	0.16	3.9±0.1	8±1	1.3	4.7	2.4
51	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	298	0.17	17±1	39±2	3.4	1.0	3.0
52[c]	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	308.3	0.17	24.1±0.4	47.0±0.4	2.6	2.2	1.8
53	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	304.4	0.18	19±1	41±1	1.7	0.4	1.4
54	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	286.3	0.17	19.9±0.01	40±1	0.7	2.4	0.8
55	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	302.6	0.18	20±1	40±1	4.5	2.1	0.8
56	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	289.9	0.18	18.1±0.3	40±1	2.7	2.7	0.8
57	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	305.5	0.18	16.6±0.3	36±1	3.2	4.2	4.7
58	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	310.4	0.17	21.3±0.2	38.2±0.3	0.8	0.2	0.7
59	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	311.1	0.18	20.6±0.1	42±1	0.9	1.4	0.7
60[d]	1.0 : 7.0	$HClO_4$ (3.0 equiv.)	60	60°C, 24 h	296.5	0.18	23.3±0.4	44±1	2.4	2.8	1.9

Continuation of Table S8

Entry	Monomer ratio[a]	Template	Milling time, min	Aging	Solids, mg	η, µL/mg	HPLC yield, %		*RSD* of peak area, %		
							mixHC[8]	cycHC[8]	mixHC[8]	cycHC[8]	TPM
61	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	309.1	0.19	33.9±0.1	37±1	0.5	1.5	0.8
62	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	308.8	0.19	34.6±0.4	40.9±0.3	3.1	2.5	2.0
63	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	306.9	0.20	27±2	32±1	6.0[b]	4.1	0.2
64	1.0 : 7.0	HPF_6 (2.0 equiv.), $AgPF_6$ (1.0 equiv.)	60	60°C, 24 h	306.5	0.19	29.8±0.4	36.4±0.2	0.6	0.9	1.3
65	1.0 : 7.0	HPF_6 (2.0 equiv.), $[Cu(CH_3CN)_4]PF_6$ (1.0 equiv.)	60	60°C, 24 h	302.6	0.18	31.3±0.1	43±1	1.7	0.9	1.8
66	1.0 : 7.0	HPF_6 (2.0 equiv.), $[Cu(CH_3CN)_4]$PF6 (1.0 equiv.)	60	60°C, 24 h	301.5	0.18	30.8±0.3	45±1	3.0	2.1	3.4
67	1.0 : 7.0	HPF_6 (2.4 equiv.)	60	60°C, 24 h	273.2	0.26	27.5±0.2	36±1	1.6	2.0	0.8
68	1.0 : 7.0	HPF_6 (3.0 equiv.)	60	60°C, 24 h	273.7	0.33	27.7±0.3	32.8±0.1	1.4	2.8	2.4
69	1.0 : 7.0	$HClO_4$ (3.0 equiv.), $CuClO_4$ (1.0 equiv.)	60	60°C, 24 h	291.5	0.14	26±1	39.5±0.3	1.6	0.6	0.2
70	1.0 : 7.0	$HClO_4$ (3.0 equiv.), $CuClO_4$ (1.0 equiv.)	60	60°C, 24 h	293.3	0.13	25.7±0.1	38±1	0.7	1.7	0.8
71	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	306.7	0.19	31.5±0.2	38±1	1.3	2.6	1.5
72	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.1	0.19	34.4±0.2	36.5±0.2	0.9	1.9	1.4
73	1.0 : 5.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	310.2	0.19	34±1	30±1	2.5	2.5	0.7
74	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.2	0.20	41±1	38.1±0.2	0.7	1.3	1.4
75	1.0 : 15.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	300.7	0.20	27±1	61.8±0.4	2.0	0.5	0.2
76	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	5	60°C, 24 h	307.3	0.20	15.1±0.4	55±3	1.5	4.1	1.8
77	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	10	60°C, 24 h	307.9	0.19	24±1	36.7±0.2	1.7	1.1	1.7
78	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	20	60°C, 24 h	307.9	0.19	37.4±0.3	39±1	3.1	1.2	2.5
79	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	30	60°C, 24 h	309.7	0.19	36.3±0.4	34.1±0.3	0.9	1.1	0.3
80	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	45	60°C, 24 h	307.9	0.19	23.3±0.3	25±1	1.2	4.2	0.4
81	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	309.3	0.19	36.3±0.3	36±1	1.2	1.2	0.6
82	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	5	60°C, 24 h	307.7	0.19	16±1	52±3	3.9	3.9	1.3
83	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	10	60°C, 24 h	306.9	0.20	23.9±0.2	38±1	1.6	1.7	0.8
84	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	20	60°C, 24 h	307	0.20	37±1	40±1	1.0	1.3	1.1
85	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	30	60°C, 24 h	308.1	0.19	28.2±0.4	29±1	1.6	4.7	2.9
86	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	45	60°C, 24 h	308.5	0.19	24±1	26±1	1.9	2.1	0.9
87	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	305.6	0.20	37±1	41.0±0.3	3.5	1.5	2.2
88	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	90	60°C, 24 h	307	0.20	34.5±0.1	32.5±0.4	2.6	2.2	2.4
89	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	90	60°C, 24 h	309.2	0.19	33±1	31.0±0.3	1.3	1.9	1.5
90	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	30	60°C, 24 h	308.8	0.19	30±1	30±1	2.5	3.2	1.5

Continuation of Table S8

Entry	Monomer ratio	Template	Milling time, min	Aging	Solids, mg	η, µL/mg	HPLC yield, %		*RSD* of peak area, %		
							mixHC[8]	cycHC[8]	mixHC[8]	cycHC[8]	TPM
91	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	45	60°C, 24 h	306.9	0.20	33±1	34±1	1.8	2.3	0.8
92	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 3 h	307.6	0.20	38.6±0.4	33.3±0.2	1.0	1.6	1.3
93	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 3 h	307.8	0.19	38±2	35.6±0.4	3.5	2.6	2.2
94	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 6 h	308.3	0.19	36.9±0.1	35±1	0.3	2.0	0.1
95	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 6 h	307.4	0.20	38.0±0.2	35±1	0.6	2.0	1.2
96	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 12 h	306.9	0.20	32±1	34±1	0.6	0.2	2.5
97	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 12 h	308	0.19	31±1	32±1	0.5	0.8	3.1
98	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	308	0.19	15.8±0.3	57.6±0.1	4.1	3.0	3.0
99	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.2	0.20	24±1	41.6±0.3	2.8	2.1	1.5
100	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	306.9	0.20	36±1	38±1	1.9	1.4	1.9
101	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.5	0.20	36±1	36±1	2.8	1.3	3.6
102	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.7	0.19	26±1	29±1	2.6	2.4	3.8
103	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	60	60°C, 24 h	307.1	0.20	34.9±0.2	36±1	0.7	0.6	1.3
104[c]	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	20	60°C, 3 h	303.8	0.19	30±1	32±1	5.0	3.8	3.2
105[c]	1.0 : 7.0	HPF_6 (2.0 equiv.), KPF_6 (1.0 equiv.)	20	60°C, 3 h	304	0.19	30±1	32±1	4.5	3.9	3.3

[a] Biotin : cyclohexa-1,2-diylurea molar ratio. [b] Outliers. [c] Number of replicates: n=9. [d] Number of replicates: n=6.

Accuracy

The relative spike recovery (R_i, %) was calculated via formula (3):

$$R_i = \frac{S_i}{S_{Ti}} \cdot 100 = \frac{S_i}{S_{RM,i} + S_{\%i}} \cdot 100 \quad (3)$$

where S_i – peak area of the analyte i in the spiked sample, mAU·s; S_{Ti} – theoretical value of the analyte i peak area in the spiked sample, mAU·s; $S_{RM,i}$ – analyte i peak area in the non-spiked sample of the crude reaction mixture, mAU·s; $S_{\%i}$ – peak area of the spike addition of the analyte i.

The bias of recovery (δ_{Ri}, %) was expressed as (4):

$$\delta_{Ri} = |100 - R_i| \quad (4)$$

Table S9. Accuracy studies (TPM)

Sample	S_i[a], **mAU·s**	S_{Ti}, **mAU·s**	R_i, **%**	δ_{Ri}, **%**
Reaction mixture (contains 10 mg TPM)	608±2	–	–	–
10% TPM addition	63.3±0.3	–	–	–
5% TPM addition	33±1	–	–	–
2.5% TPM addition	17±2	–	–	–
Reaction mixture + 10% TPM	685±3	671.3	102.1	2.1
Reaction mixture + 5% TPM	659±1	641	102.7	2.7
Reaction mixture + 2.5% TPM	627±2	625	100.3	0.3

[a] The mean area values are provided as the average of 3 replicas ± standard deviation between parallel results.

Table S10. Accuracy studies (macrocycles)

Sample	S_i[a], **mAU·s**	S_{Ti}, **mAU·s**	R_i, **%**	δ_{Ri}, **%**
MixHC[8] in reaction mixture	365±1	–	–	–
23% mixHC[8] addition	83±2	–	–	–
30% mixHC[8] addition	109±3	–	–	–
51% mixHC[8] addition	185±4	–	–	–
Reaction mixture + 23% mixHC[8]	459±2	448	102.5	2.5
Reaction mixture + 30% mixHC[8]	484±3	474	102.1	2.1
Reaction mixture + 50% mixHC[8]	547±9	550	99.5	0.5
CycHC[8] in reaction mixture	393±6	–	–	–
22% cycHC[8] addition	85±2	–	–	–
27% cycHC[8] addition	107±3	–	–	–
37% cycHC[8] addition	147±6	–	–	–
Reaction mixture + 22% cycHC[8]	468±6	477	98.0	2.0
Reaction mixture + 27% cycHC[8]	510±4	499	102.2	2.2
Reaction mixture + 37% cycHC[8]	540±9	539	100.0	0

[a] The mean area values are provided as the average of 3 replicas ± standard deviation between parallel results.

Reproducibility

Table S11. Reproducibility of yield estimation (2 parallel reactions, 9 samples from each)

Entry	Product	*Yield*$_i$, %									*Yield*$_{mean}$, %	*RSD*, %
1[a]	mixHC[8]	30	33	28	30	31	30	31	30	32	31±1	4
	cycHC[8]	32	32	31	31	32	31	33	32	31	32±1	3
2[b]	mixHC[8]	31	30	31	31	29	29	30	30	29	30±1	3
	cycHC[8]	32	32	33	32	31	32	31	30	31	32±1	3

[a] Crude mixture combined using a spatula prior to sampling. [b] Crude mixture not combined prior to sampling.

References

1 M. Fomitšenko, E. Shmatova, M. Öeren, I. Järving and R. Aav, *Supramolecular Chemistry*, 2014, **26**, 698–703.

2 E. Suut-Tuule, T. Jarg, P. Tikker, K.-M. Lootus, J. Martõnova, R. Reitalu, L. Ustrnul, J. S. Ward, V. Rjabovs, K. Shubin, J. V. Nallaparaju, M. Vendelin, S. Preis, M. Öeren, K. Rissanen, D. Kananovich and R. Aav, *Cell Rep. Phys. Sci.*, 2024, 102161.