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Glucuronidation of bile acids under flow conditions: design of experiments and Koenigs–Knorr reaction optimization†

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An efficient method for the C_3-glucuronidation of bile acids is developed under flow conditions. A modular mesoreactor assisted flow set-up was combined with statistical design of experiments to speed up the optimization of the Koenigs–Knorr reaction in terms of yield, regioselectivity, costs, as well as technical and practical standpoints. Using the optimal conditions, selective glucuronidation of naturally occurring bile acids was successfully achieved offering a new, valuable route to C_3-glucuronidated bile acids useful for biological, diagnostic and PK/ADMET investigations.

Introduction

Glucuronidation is a minor but well established pathway in normal bile acid (BA) metabolism while it represents a major route for detoxification under pathological conditions.[1] This phase II metabolic process consists of the conjugation reaction with glucuronic acid that turns free BAs into higher soluble metabolites which are then rapidly excreted into bile or eliminated in urine.[2] In humans, the most abundant glucuronides are glucuronide of chenodeoxycholic and lithocholic acid, whose concentration can significantly increase in patients affected by liver diseases such as cholestasis.[1,3–5] The ready availability of pure compounds is therefore of crucial importance to determine their levels in urine and plasma as biomarkers for clinical diagnosis and response to therapy. Not less importantly, BA glucuronides are needed in pharmacokinetic evaluations of BA-based clinical candidates to assess their relative concentration and biodistribution within metabolic tissues and organs.[6]

To date, only a few methods have been described for the preparation of C_3-BA glucuronides. These include extractive methodologies and isolation from biological fluids,[3] enzymatic preparations,[7] and chemical syntheses.[8,9] Whilst the use of enzymatic and extractive methodologies remains elusive, the Koenigs–Knorr reaction represents the most used chemical transformation to achieve *O*-glucuronidated steroids.[8,9] It is based on the reaction of the aglycone with an opportunely protected glucuronyl halide (chloride or bromide) in the presence of Lewis acids or heavy metal salts such as silver and cadmium salts. Anhydrous benzene, toluene, acetonitrile, dichloromethane or ethers are the solvents of choice, and the addition of molecular sieves or adsorbents such as drierite is recommended to remove any trace of water and to avoid the hydrolysis of susceptible glucuronyl halide. It is noteworthy that the few examples of C_3-glucuronidation of BAs by the Koenigs–Knorr reaction make use of $CdCO_3$ as the coupling agent and suffer from several drawbacks including low yields, the use of drastic reaction conditions, long reaction times and tedious purifications.[10] Furthermore, extra steps of protection–deprotection are generally required in order to avoid the glucuronidation of hydroxy groups at C_7 and C_{12} positions of the steroid scaffold.

Based on these premises and as a continuation of our interest in the preparation of BAs and metabolites using automated flow synthesizers,[11] we report the development of a new synthetic methodology for the selective C_3-glucuronidation of BAs under flow conditions. In this task, statistical design of experiments (DoE) was instrumental to improve the efficiency of the Koenigs–Knorr reaction in terms of yield, regioselectivity, costs, as well as technical and practical advantages.

Results and discussion

DoE-assisted Koenigs–Knorr reaction optimization under flow conditions

The optimization of the Koenigs–Knorr reaction to prepare C_3-glucuronidated BAs under continuous flow was based on

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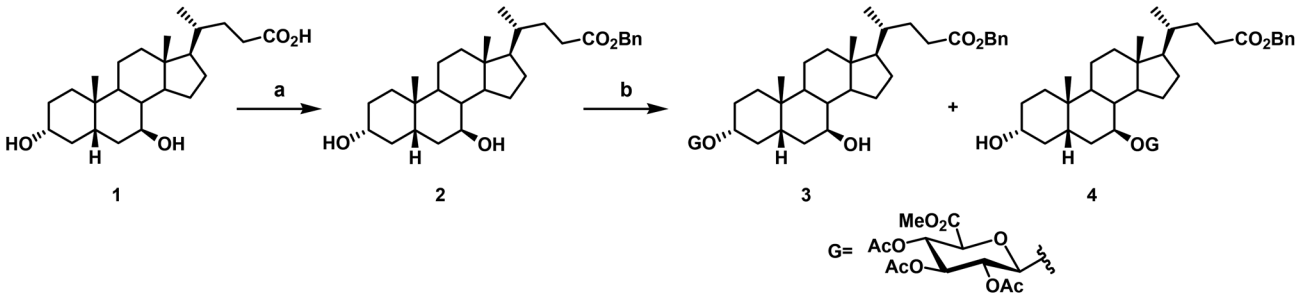


Scheme 1 Model reaction. *Reaction conditions*: (a) benzyl bromide, Cs_2CO_3, CH_3CN, reflux, 4 h, 82%; (b) Fetizon's reagent (28% loading), molecular sieves (4 Å, 325 mesh), methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**), toluene, 25 °C, 48 h, **3**: 58%, **4**: 40%.

the study of four consequential phases including (a) the choice of the model reaction, (b) validation of the analytical method for the quantitative determination of the reaction yield, (c) design of a convenient flow set-up, and (d) optimization of the reaction conditions by the DoE study. Once the optimal flow set-up and experimental conditions were established, the scope was extended to a set of natural BAs assessing at the same time the versatility and efficiency of the method.

Model reaction. Benzyl ursodeoxycholate (**2**) was selected as the model BA substrate for DoE reaction optimization. This decision was made taking into consideration the higher reactivity of the $C_7\beta$ hydroxy group of ursodeoxycholic acid (UDCA, **1**) towards the glucuronidation reaction in order to enhance the challenge to get the maximum information on the regioselectivity issue of the reaction. Esterification with the benzyl group was profitable to increase the solubility of the BA scaffold in toluene, the solvent of choice for the Koenigs–Knorr reaction, and to guarantee the easy in-line UV detection of biliary species within the reaction mixtures. Thus, UDCA (**1**) was refluxed with benzyl bromide in the presence of Cs_2CO_3 in acetonitrile to furnish the desired ester **2** in 82% yield (Scheme 1).[12]

Among the various catalysts previously employed in the Koenigs–Knorr coupling reactions,[8] insoluble silver salts as Ag_2O and Ag_2CO_3 were particularly attractive as heterogeneous reactants. In our case, we made use of a Celite-supported silver carbonate namely Fetizon's reagent (28% loading), which was easily prepared by treating Celite with a solution of $AgNO_3$ and Na_2CO_3 in water.[13] Methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**) was then selected as the glucuronyl donor.[14]

HPLC analysis. The employment of flow chemistry in the process optimization of the BA metabolites required the validation of a HPLC method for the determination of reaction yields as well as of the purity grade of the synthesized conjugates.[11,15] In this context, a unique HPLC method was successfully established and validated for benzyl ursodeoxycholate (**2**), as well as for the corresponding C_3- and C_7-glucuronides **3** and **4**. Accordingly, an evaporative light scattering detector (ELSD) was profitably utilized for the analysis of such steroidal species.[16] All runs were contemporarily carried out on the ester **2** and conjugated species **3** and **4**. The different ELSD response of the free **2** and the corresponding conjugated BAs **3** and **4** imposed to build-up separate calibration curves. In all the cases, very good precision and accuracy (evaluated both in the short and long period) along with remarkably low LOD and LOQ values were obtained (see ESI†).

Flow set-up. Flow experiments were performed in a flow mesoreactor equipped with a loop injection system (2 mL), a pump dedicated to a reservoir of organic solvent, a Omnifit PEEK column (L × ID 150 mm × 6.6 mm) packed with the Fetizon's reagent and molecular sieves (1 : 1, w/w) and fixed in a reactor heater, a back pressure regulator (BPR) (100 psi), and a UV detector (Fig. 1) useful to define the residence time and

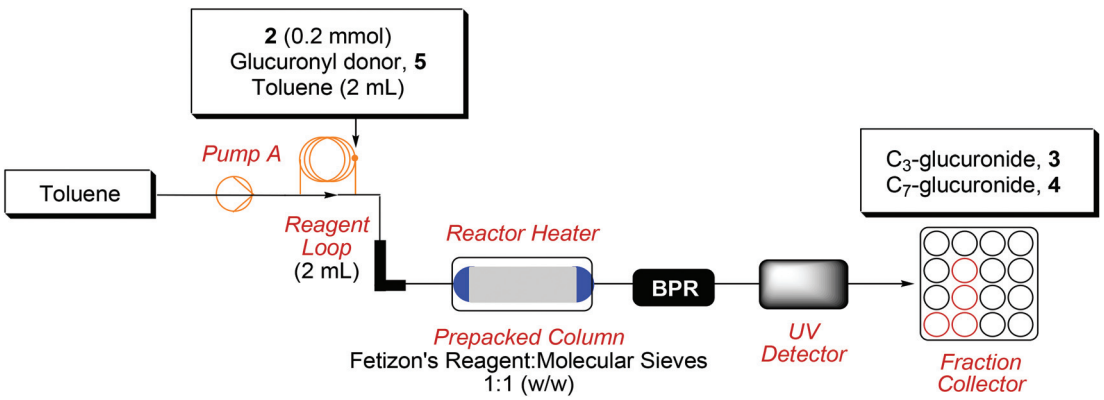


Fig. 1 Flow set-up used during the optimization of reaction conditions.

Table 1 Variable settings for Koenigs–Knorr reaction optimization

Factor	Units	Range
Temperature (*A*)	°C	25–80
Flow rate (*B*)	mL min^{-1}	0.1–0.4
Glucuronyl donor **5** (*C*)	equivalents	1.2–3

Table 2 Central composite design: experimental matrix and measured responses[a]

Run	Type	*A* (°C)	*B* (mL min^{-1})	*C* (equiv.)	Y^b (%)	Z^b (%)
1	Axial	80	0.25	2.1	73	50
2	Axial	53	0.25	1.2	70	47
3	Factorial	69	0.34	1.6	75	54
4	Factorial	36	0.16	2.6	97	72
5	Factorial	36	0.34	1.6	31	20
6	Factorial	36	0.16	1.6	79	52
7	Factorial	69	0.34	2.6	83	57
8	Center	53	0.25	2.1	87	59
9	Axial	25	0.25	2.1	45	29
10	Factorial	69	0.16	2.6	72	49
11	Axial	53	0.4	2.1	68	45
12	Factorial	36	0.34	2.6	66	44
13	Axial	53	0.25	3	84	57
14	Center	53	0.25	2.1	78	51
15	Center	53	0.25	2.1	80	52
16	Axial	53	0.10	2.1	85	57
17	Factorial	69	0.16	1.6	68	46
18	Center	53	0.25	2.1	83	54
19	Center	53	0.25	2.1	84	55

[a] All reactions were conducted according to Fig. 1. *Reagents and conditions*: **2** (0.2 mmol, 0.1 M in toluene), glucuronyl halide (**5** in toluene), a reactor packed with Fetizon's reagent (28% Ag_2CO_3, 5 equiv.) and molecular sieves (4 Å, 325 mesh) in a 1 : 1 ratio (w/w), toluene. [b] Determined by HPLC analysis of the crude reaction mixtures.

relative distribution in the packed bed reactor. During the optimization of experimental conditions, reactions were performed by loop injection of solutions of **2** (0.2 mmol, 0.1 M) and glucuronyl donor **5** in toluene. After the injection and the switching of the valve through the loop, the solution was pumped into the reaction column packed with the Fetizon's reagent and warmed at the selected temperature. The output was collected in a fraction collector and analyzed by quantitative HPLC analysis.

DoE-assisted reaction optimization. With the aim to find the experimental conditions that would ensure maximum conversion (*Y*) and reaction yield (*Z*), a response surface design was adopted to explore the effect of those variables considered important in the process.[17] These include the temperature (*A*), the flow rate (*B*), and the stoichiometry of the glucuronyl donor **5** (*C*) whose relative investigation range is shown in Table 1.

The reaction space was thus screened by a central composite design (CCD) composed of a set of 14 experiments plus five replicates at the central point. The results and responses are reported in Table 2.

The mathematical models, as defined by eqn (1) and (2), and relative response-surfaces (Fig. 2) were obtained by fitting the acquired data into the following quadratic equation:

$$y = \beta_0 + \beta_A A + \beta_B B + \beta_C C + \beta\beta_{AB} AB + \beta\beta_{AC} AC + \beta\beta_{BC} BC + \beta_A{}^2 A^2 + \beta_B{}^2 B^2 + \beta_C{}^2 C^2$$

The measured response is indicated as *y*, β_0 is the value of the function at the centre point while coefficients (β_i, β_{ij}, $\beta_i{}^2$) represent the weight of related variables (x_i, x_{ij}, $x_i{}^2$) on the result. The statistical significance was confirmed by the analysis of the variance as shown in Table 3.

As emerged from the analysis of equation coefficients, though the temperature apparently had a positive (linear) effect (β_A) on the reaction yield (*Z*) (Table 3), the high weight of the interaction term β_{AB} together with the negative value of the quadratic $\beta_A{}^2$ suggested that an increase of the temperature should be accompanied by a relative increase of the flow rate. Moreover, the term related to sugar equivalents (β_C) was likely to have an opposite effect when combined with high temperatures as confirmed by the negative value of the interaction coefficient (β_{AC}). In other words, the thermal (in)stability of the glucuronyl donor **5** affects the reaction outcome in relationship with the temperature applied especially at lower flow rates. This dependence of measured responses (*Y*, *Z*) with respect to the experimental variables (*A*, *B* and *C*) is illustrated in the contour plots and 3D surfaces (Fig. 2), generated from the corresponding quadratic models.

Mathematical models were then interrogated to examine the effect on conversion (*Y*) and reaction yield (*Z*) in three different scenarios suggesting the best outcome (*Y* = 97%, *Z* = 68%) using 2.6 equivalents of **5** at 38 °C and 0.16 mL min^{-1}. Remarkably, the predicted and experimental yields showed a high degree of correlation, thus demonstrating a good statistical significance and the robustness of our model (Table 4).

C_3-glucuronidation of bile acid derivatives under flow conditions

With the scope to explore the versatility of our optimized methodology, the reaction was applied to naturally occurring BAs including cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid. Thus, each BA was initially transformed into the corresponding benzyl ester derivative (**6–9**) and then subjected to flow glucuronidation (Fig. 3, Table 5).

As shown in Table 5, the desired conjugates **10–13** were isolated in good yields and with a high degree of regioselectivity as no C_7- or C_{12}-glucuronidation was detected. The obtained regioselectivity can be ascribed to the heterogeneous nature of the reaction.[13c] Indeed, absorption of BAs on the solid surface of Fetizon's reagent may play a critical role; it can be speculated that the absorption geometry and disposition of benzyl esters **6–9** on Celite make the hydroxy group at the $C_3\alpha$ position more reactive than C_7 and C_{12} counterparts.[13b] Furthermore, steric and conformational factors resulting from the diverse polyhydroxylation of the biliary derivatives could alter the absorption geometry on Celite affecting the reaction rate. Remarkably, the reaction proceeds with high chemoselectivity; although Fetizon's reagent is a well known oxidizing agent, the

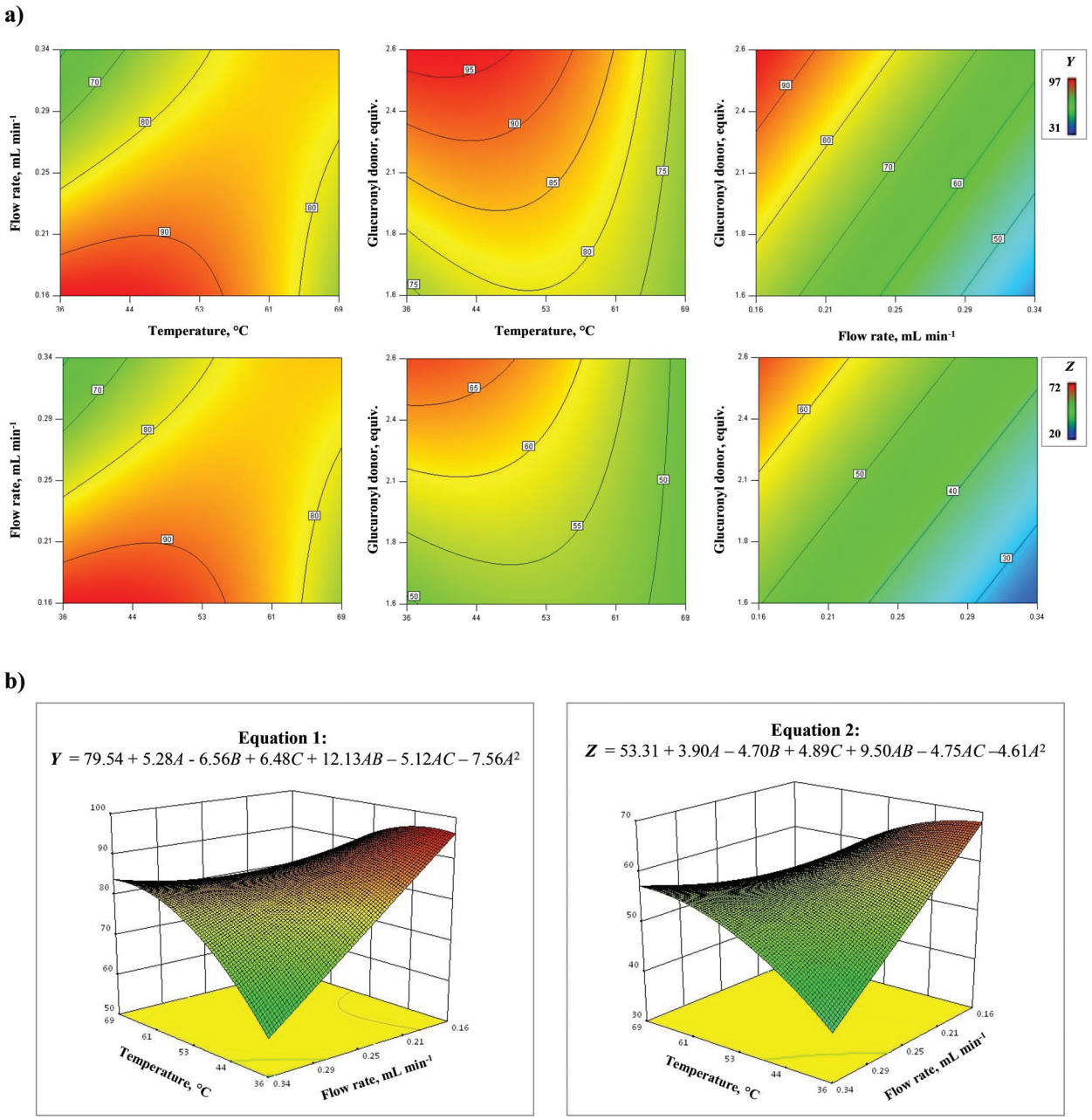


Fig. 2 (a) Contour plots for glucuronyl donor **5** (2.6 equiv.), flow rate (0.16 mL min^{-1}), temperature (38 °C). (b) Response surfaces for 2.6 equiv. glucuronyl donor **5** with respect to conversion (*Y*) and reaction yield (*Z*).

presence of 3-keto-derivatives was never observed in the crude reaction mixtures. The sole by-products formed were mono- and poly-*O*-acetylated compounds resulting from the dioxolane ion and orthoester rearrangement (Scheme 2).[18]

Conclusions

The role of BAs in drug discovery appears to be set to expand enormously. A number of BA derivatives has been used as valuable pharmacological tools to explore the biological and therapeutic relevance of nuclear and membrane receptors, with some of them having entered into preclinical and clinical trials.[19] As a result, synthetic chemists active in the field are often asked to target BAs more quickly and *via* synthetic routes capable of delivering rapid multiple synthesis and supporting the following scale-up.

In this framework, as a continuation of our interest in flow technology,[11,20] we demonstrate how the combination of flow synthesizers and statistical DoE can be instrumental in the optimization of the Koenigs–Knorr reaction, resulting in a convenient access to C_3-glucuronidated BAs, crucial metabolites for clinical analysis and diagnosis, as well as for biological and pharmacokinetic evaluations. In particular, using flow devices

Table 3 ANOVA table and associated statistics related to the response surface model for observables *Y* and *Z*

	Y					*Z*				
Source	Sum of squares	Degrees of freedom	Mean square	*p*-value Prob > *F*	β_i[a]	Sum of squares	Degrees of freedom	Mean square	*p*-value Prob > *F*	β_i[a]
Model	3740.18	6	623.36	<0.0001	79.54	2040.73	6	340.12	<0.0001	53.31
A	380.54	1	380.54	0.0041	5.28	208.16	1	208.16	0.0022	3.90
B	587.72	1	587.72	0.0009	−6.56	301.63	1	301.63	0.0005	−4.70
C	574.09	1	574.09	0.0010	6.48	326.92	1	326.92	0.0004	4.89
AB	1176.13	1	1176.13	<0.0001	12.13	722.00	1	722.00	<0.0001	9.50
AC	210.12	1	210.12	0.0221	−5.12	180.50	1	180.50	0.0035	−4.75
A2	811.58	1	811.58	0.0002	−7.56	301.53	1	301.53	0.0005	−4.61
Residual	365.61	12	30.47			165.27	12	13.77		
Lack of fit	316.41	8	39.55	0.1371		126.47	8	15.81	0.3351	
Pure error	49.20	4	12.30			38.80	4	9.70		
Cor total	4105.79	18				2206.00	18			
	R^2 = 0.9110, Adj R^2 = 0.8664, Pred R^2 = 0.7041					R^2 = 0.9251, Adj R^2 = 0.8876, Pred R^2 = 0.7600				
	PRESS = 1214.91, Adeq Precision = 18.084					PRESS = 529.53, Adeq Precision = 21.169				

[a] β_i = Estimated regression coded coefficients.

Table 4 Comparison of predicted and experimental yields under different reaction conditions[a]

		Y (%)		*Z*(%)	
Targets	Suggested conditions	Predicted	Experimental[b]	Predicted	Experimental[b]
Maximize *Y*, *Z* *A*, *B*, C in range	*A*: 38 °C *B*: 0.16 mL min^{-1} C: 2.6 equiv.	97	95	68	65
Maximize *Y*, *Z*, *B* Minimize *C* *A* in range	*A*: 49 °C *B*: 0.20 mL min^{-1} *C*: 2.3 equiv.	86	89	58	60
Maximize *Y*, *Z* Minimize *C* *A* and *B* in range	*A*: 50 °C *B*: 0.16 mL min^{-1} *C*: 1.7 equiv.	85	85	55	56

[a] All reactions were conducted according to Fig. 1. *Reagents and conditions*: **2** (0.2 mmol, 0.1 M in toluene), glucuronyl halide (**5** in toluene), a reactor packed with Fetizon's reagent (28% Ag_2CO_3, 5 equiv.) and molecular sieves (4 Å, 325 mesh) in a 1 : 1 ratio (w/w), toluene. [b] Determined by HPLC analysis of the crude reaction mixtures.

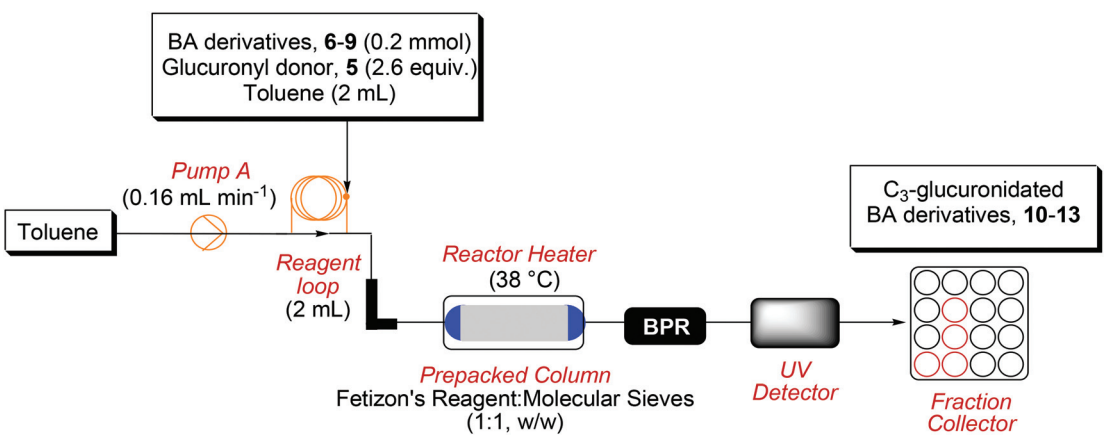
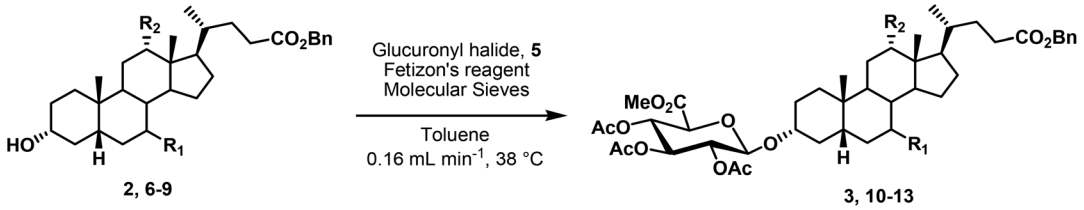


Fig. 3 General scheme used for the synthesis of C_3-glucuronidated bile acids.

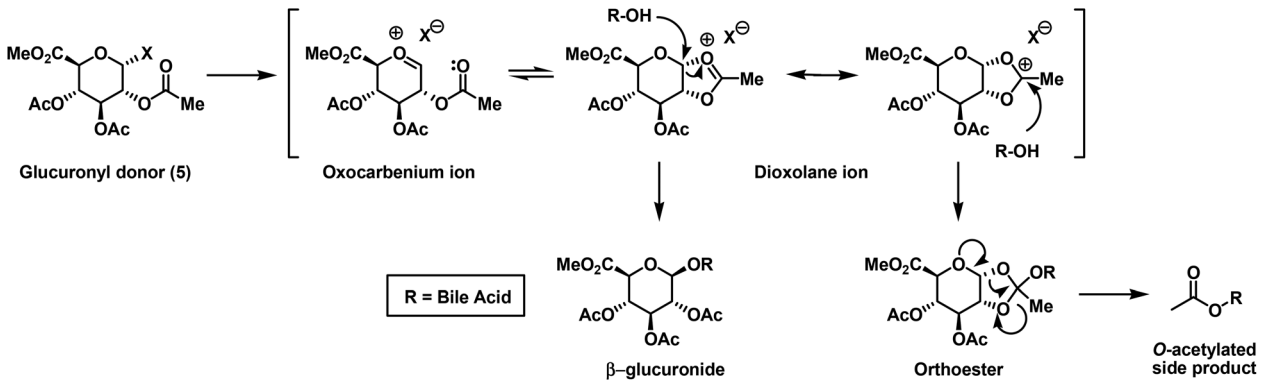
for the rapid and accurate investigation of the influence on the reaction outcome of selected experimental parameters, a DoE screening of the relevant variables was carried out for a response surface process optimization. Mathematical models thus obtained displayed a good predictivity within the explored chemical space leading to a robust and reproducible flow pro-

Table 5 Glucuronidation of bile acid derivatives in flow condition mode[a]



Index	Bile acid derivative	R_1	R_2	Product, yield[b]
1	Benzyl ursodeoxycholate (2)	β-OH	–H	**3**, 69%
2	Benzyl cholate (**6**)	α-OH	–OH	**10**, 77%
3	Benzyl chenodeoxycholate (7)	α-OH	–H	**11**, 65%
4	Benzyl deoxycholate (**8**)	–H	–OH	**12**, 77%
5	Benzyl lithocholate (**9**)	–H	–H	**13**, 70%

[a] All reactions were conducted according to Fig. 3. [b] Isolated yield.



Scheme 2 Mechanism for the formation of 3-*O*-acetylated side products.

cedure characterized by better yields and reduced reaction time with respect to previously reported batch approaches.[10] Moreover, using the optimal conditions, selective glucuronidation at the C_3 position of the steroidal body was obtained, as no C_7 or C_{12} conjugates could be detected except for UDCA derivative **2**. Future research could lead to a more intensified process by, as an example, the use of an improved metal-based coupling agent.

Experimental section

General methods

^{1}H-NMR spectra were recorded at 400 MHz, and ^{13}C-NMR spectra were recorded at 100.6 MHz using the solvents indicated below. Chemical shifts are reported in ppm. The abbreviations used are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; m, multiplet; pst, pseudo-triplet. The DoE was carried out with the support of the software Design-Expert 8.0.7.1. All flow experiments were performed using a commercially available Vapourtec R2+/R4 module. TLC was performed on aluminium backed silica plates (silica gel 60 F254). HPLC measurements were made on a Shimadzu LC-20A Prominence equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector and a Rheodyne 7725i injector with a 20 μL stainless steel loop. A Varian 385-LC ELSD was utilized for the analyses of investigated compounds. The analog-to-digital conversion of the output signal from the ELSD was allowed using a common interface device. The adopted ELSD conditions for the analysis of all experimental design runs were 90 °C nebulization temperature, 75 °C evaporation temperature, 1 mL min^{-1} gas flow rate (air) and 1.0 as the gain factor. A Grace-Smart RP18 column 250 × 4.6 mm I.D., 5 μm, 100 Å was used as the analytical column while the eluent was H_2O–CH_3CN 30/70 (v/v). The column temperature (25 °C) was controlled through a Grace heather/chiller thermostat. The final products were purified by flash chromatography on silica gel (0.040–0.063 mm). Melting points were determined by the capillary method using a Buchi 535 instrument and they were not corrected. Methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide (**5**) was prepared as previously reported.[14]

General method for the synthesis of benzyl esters 2, 6–9[12]

Cs_2CO_3 (1.22 g, 3.75 mmol) and benzyl bromide (1.49 mL, 12.5 mmol) were added to a solution of BA (2.5 mmol) in

CH_3CN (16 mL). The resulting mixture was refluxed for 4 h. The suspension was cooled to room temperature, filtered and concentrated under reduced pressure. The residue was treated with a saturated solution of $NaHCO_3$ and extracted with EtOAc. The collected organic layers were washed with H_2O and brine, dried over anhydrous Na_2SO_4 and concentrated under *vacuum*. The crude mixture was purified by silica gel flash chromatography using CH_2Cl_2–MeOH as an eluting solvent system.

Benzyl 3α,7β-dihydroxy-5β-cholan-24-oate (**2**) was obtained in 82% isolated yield as a white solid, mp: 140.4–141.4 °C. δ_H (400 MHz, d_6-DMSO) 0.60 (3H, s, C(18)H_3), 0.89–0.92 (6H, m, C(19)H_3, C(21)H_3), 3.29–3.34 (2H, m, C(3)H, C(7)H), 3.88 (1H, d, J = 6.7 Hz, C(7)OH), 4.46 (1H, d, J = 4.4 Hz, C(3)OH), 5.05–5.13 (2H, m, CH_2Ph), 7.33–7.38 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 11.9, 18.2, 20.8, 23.3, 26.6, 28.1, 30.2, 30.6, 30.7, 33.7 (2×), 34.7, 34.8, 37.3, 37.7, 38.7, 42.2, 42.9, 43.1, 54.6, 55.8, 65.3, 69.4, 69.7, 127.9 (3×), 128.4 (2×), 136.3, 173.1.

Benzyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (**6**) was obtained in 82% isolated yield as a white solid, mp: 65–68 °C. δ_H (400 MHz, d_6-DMSO) 0.55 (3H, s, C(18)H_3), 0.80 (3H, s, C(19)H_3), 0.91 (3H, d, J = 5.6 Hz, C(21)H_3), 3.15–3.18 (1H, m, C(3)H), 3.60 (1H, s, C(7)H), 3.76 (1H, s, C(12)H), 4.04 (1H, d, J = 3.3 Hz, C(12)OH), 4.00 (1H, d, J = 3.4 Hz, C(7)OH), 4.10 (1H, d, J = 4.3 Hz, C(3)OH), 5.03–5.10 (2H, m, CH_2Ph), 7.31–7.36 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.7, 17.3, 23.0 (2×), 23.2, 26.6, 27.7, 28.9, 30.8, 31.1 (2×), 34.8 (2×), 35.3, 35.4, 35.7, 41.8, 41.9, 46.2, 46.5, 65.7, 66.7, 70.9, 71.4, 128.3 (3×), 128.8 (2×), 136.7, 173.6.

Benzyl 3α,7α-dihydroxy-5β-cholan-24-oate (**7**) was obtained in 80% isolated yield as a white solid, mp: 142–144 °C. δ_H (400 MHz, d_6-DMSO) 0.59 (3H, s, C(18)H_3), 0.85 (3H, s, C(19)H_3), 0.89 (3H, d, J = 6.3 Hz, C(21)H_3), 3.21–3.24 (1H, m, C(3)H), 3.64 (1H, s, C(7)H), 4.11 (1H, d, J = 3.3 Hz, C(7)OH), 4.31 (1H, d, J = 4.7 Hz, C(3)OH), 5.07–5.10 (2H, m, CH_2Ph), 7.33–7.39 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 11.6, 18.1, 20.3, 22.7, 23.2, 27.8, 30.5, 30.6, 30.7, 32.3, 34.8 (2×), 34.9 (2×), 35.3, 41.4, 42.0, 50.0, 55.5, 65.3, 66.2, 70.4, 128.0 (3×), 128.5 (2×), 136.3, 173.2.

Benzyl 3α,12α-dihydroxy-5β-cholan-24-oate (**8**) was obtained in 79% isolated yield as a white solid, mp: 125–128 °C. δ_H (400 MHz, d_6-DMSO) 0.56 (3H, s, C(18)H_3), 0.84 (3H, s, C(19)H_3), 0.90 (3H, d, J = 6.0 Hz, C(21)H_3), 3.34–3.37 (1H, m, C(3)H), 3.76 (1H, s, C(12)H), 4.20 (1H, d, J = 4 Hz, C(12)OH), 4.46 (1H, d, J = 4.3 Hz, C(3)OH), 5.05–5.08 (2H, m, CH_2Ph), 7.33–7.36 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.4, 16.8, 23.1, 23.5, 26.1, 27.0, 27.1, 28.6, 30.2, 30.7, 32.9, 33.8, 34.9, 35.1, 35.6, 36.3, 41.6, 46.0, 46.1, 47.4, 62.8, 65.3, 69.9, 71.0, 127.9 (2×), 128.0, 128.4 (2×), 136.3, 173.1.

Benzyl 3α-hydroxy-5β-cholan-24-oate (**9**) was obtained in 85% isolated yield as a white solid, mp: 122–124 °C. δ_H (400 MHz, d_6-DMSO) 0.58 (3H, s, C(18)H_3), 0.85–0.88 (6H, m, C(19)H_3, C(21)H_3), 3.32–3.37 (1H, m, C(3)H), 4.42 (1H, d, J = 4.3 Hz, C(3)OH), 5.03–5.11 (2H, m, CH_2Ph), 7.32–7.35 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.2, 18.5, 20.8, 23.7, 24.3, 26.6, 27.3, 28.1, 30.8, 30.9, 31.0, 34.6 (2×), 35.1, 35.6, 35.8, 36.7, 42.0, 42.7 (2×), 55.9, 56.4, 65.7, 70.3, 128.4 (3×), 128.8 (2×), 136.7, 173.5.

Preparation of Fetizon's reagent[21]

Celite was washed with a solution of MeOH–HCl 37% (90 : 10, v/v), water and dried at 120 °C. The purified anhydrous Celite (6 g) was added to a mechanically stirred solution of $AgNO_3$ (3.4 g, 20 mmol) in H_2O (20 mL) and the resulting suspension was vigorously stirred for 5 min at room temperature. A solution of $Na_2CO_3{\cdot}10H_2O$ (3 g, 10.5 mmol) in H_2O (30 mL) was added dropwise and the stirring was continued in the dark for 20 min at room temperature. The resulting yellow-green precipitate was filtered and washed with H_2O up to pH 7. The solid was dried at 125 °C for 12 h and then at 80 °C under *vacuum* for 4 h. The thus obtained Fetizon's reagent contained about 28 wt% of Ag_2CO_3 (1 mmol of Ag_2CO_3 in 1 g of powder).

C_3-glucuronidation of bile acids under flow conditions

A toluene solution consisting of BA esters (**2**, **6–9**) (0.1 M) and methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyluronate bromide **5** (2.6 equiv.) was injected with a loop and pumped at 0.16 mL min^{-1} through an Omnifit PEEK column (L × I.D. 150 mm × 6 mm) packed with Fetizon's reagent (28% loading, Ag_2CO_3 5 equiv.) and molecular sieves (4 Å, 325 mesh) (1 : 1, w/w). The reactor was warmed at 38 °C and fitted with a back pressure regulator (100 psi). The output was detected by UV, collected in a fraction collector and concentrated under reduced pressure. The crude mixture was purified by silica gel flash chromatography using CH_2Cl_2–MeOH (**3**) or PET–EtOAc (**10–13**) as the eluting solvent system.

Benzyl 3α,7β-dihydroxy-5β-cholan-24-oate-3-β-D-glucuronide methyl ester-triacetate (**3**) was obtained in 65% isolated yield as a white solid, mp: 73–74 °C. δ_H (400 MHz, d_6-DMSO) 0.58 (3H, s, C18(H)$_3$), 0.86–0.88 (6H, m, C(19)H_3, C(21)H_3), 1.95 (3H, s, OCOCH_3), 1.97–1.98 (6H, m, 2 × OCOCH_3), 3.24–3.26 (1H, m, C(7)H), 3.47–3.51 (1H, m, C(3)H), 3.64 (3H, s, CO_2CH_3), 3.88 (1H, d, J = 6.7 Hz, C(7)OH), 4.42 (1H, d, J = 9.9 Hz, C(5′)H), 4.72 (1H, pst, J = 8.4 Hz, C(2′)H), 4.90–4.96 (2H, m, C(1′)H, C(4′)H), 5.03–5.11 (2H, m, CH_2Ph), 5.31 (1H, pst, J = 9.7 Hz, C(3′)H), 7.32–7.37 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.4, 18.6, 20.6, 20.7, 20.8, 21.3, 23.5, 27.0, 28.5, 30.9, 31.2, 34.1, 34.6, 35.0, 35.1, 37.8, 39.0, 42.4, 43.3, 43.5, 52.9, 54.9, 55.9, 65.7, 69.6, 69.7, 71.3, 71.4, 71.7, 79.5, 98.5, 128.4 (3×), 128.8 (2×), 136.7, 167.9, 169.4, 169.7, 169.9, 173.6.

Benzyl 3α,7β-dihydroxy-5β-cholan-24-oate-7-β-D-glucuronide methyl ester-triacetate (**4**) was obtained in 30% isolated yield as a white solid, mp: 75.7–77 °C. δ_H (400 MHz, d_6-DMSO) 0.53 (3H, s, C18(H)$_3$), 0.83–0.86 (6H, m, C(19)H_3, C(21)H_3), 1.92–1.93 (6H, m, 2 × OCOCH_3), 1.97 (3H, s, OCOCH_3), 3.29–3.33 (1H, m, C(3)H), 3.44–3.46 (1H, m, C(7)H), 3.62 (3H, s, CO_2CH_3), 4.43–4.45 (2H, m, C(5′)H, C(3)OH), 4.72 (1H, pst, J = 8.5 Hz, C(2′)H), 4.90 (1H, pst, J = 9.7 Hz, C(4′)H), 4.98 (1H, d, J = 7.8 Hz, C(1′)H), 5.02–5.09 (2H, m, CH_2Ph), 5.26 (1H, pst, J = 9.6 Hz, C(3′)H), 7.33–7.37 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.2, 18.5, 20.7 (3×), 20.9, 21.4, 23.7, 26.9, 28.5, 30.6,

31.0, 33.7, 35.0, 35.6, 37.5, 41.5, 42.3, 43.8, 52.9, 55.0, 55.4, 65.7, 69.5 (2×), 69.9, 70.9, 71.7, 72.3, 81.8, 99.8, 128.4 (3×), 128.8 (2×), 136.7, 167.9, 169.3, 169.7, 169.9, 173.6.

Benzyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate-3-β-D-glucuronide methyl ester-triacetate (**10**) was obtained in 77% isolated yield as a white solid, mp: 75–78 °C. δ_H (400 MHz, d_6-DMSO) 0.55 (3H, s, C(18)H_3), 0.80 (3H, s, C(19)H_3), 0.91 (3H, d, J = 6 Hz, C(21)H_3), 1.97 (3H, s, OCOCH_3), 1.98 (3H, s, OCOCH_3), 1.99 (3H, s, OCOCH_3), 3.32–3.34 (1H, m, C(3)H), 3.60 (1H, s, C(7)H), 3.64 (3H, s, CO_2CH_3), 3.75 (1H, s, C(12)H), 4.09 (1H, d, J = 3.2 Hz, C(12)OH), 4.13 (1H, d, J = 3.7 Hz, C(7)OH), 4.41 (1H, d, J = 9.9 Hz, C(5′)H), 4.71 (1H, dd, J = 8.0, 9.6 Hz, C(2′)H), 4.89–4.95 (2H, m, C(1′)H, C(4′)H), 5.03–5.10 (2H, m, CH_2Ph), 5.30 (1H, pst, J = 9.6 Hz, C(3′)H), 7.33–7.37 (5H, m, C_6H_5). δ_C (100.6 MHz, DMSO-d_6) 12.3, 16.9, 20.3, 20.4, 20.5 (2×), 22.5, 22.8, 26.2, 26.6, 28.5, 30.7, 30.8, 34.4 (2×), 34.7 (2×), 34.9, 41.3, 41.5, 45.8 (2×), 46.1, 52.6, 65.4, 66.2, 69.4, 71.0, 71.4, 79.4, 97.9, 128.0 (2×), 128.1, 128.5 (2×), 136.4, 167.6, 169.1, 169.4, 169.6, 173.3.

Benzyl 3α,7α-dihydroxy-5β-cholan-24-oate-3-β-D-glucuronide methyl ester-triacetate (**11**) was obtained in 69% isolated yield as a white solid, mp: 146–148 °C. δ_H (400 MHz, d_6-DMSO) 0.57 (3H, s, C(18)H_3), 0.83 (3H, s, C(19)H_3), 0.86 (3H, d, J = 6.2 Hz, C(21)H_3), 1.95 (3H, s, OCOCH_3), 1.97 (3H, s, OCOCH_3), 1.98 (3H, s, OCOCH_3), 3.33–3.37 (1H, m, C(3)H), 3.61 (1H, s, C(7)H), 3.63 (3H, s, CO_2CH_3), 4.17 (1H, d, J = 3.2 Hz, C(7)OH), 4.42 (1H, d, J = 9.9 Hz, C(5′)H), 4.71 (1H, dd, J = 7.9, 9.6 Hz, C(2′)H), 4.89–4.95 (2H, m, C(1′)H, C(4′)H), 5.03–5.11 (2H, m, CH_2Ph), 5.31 (1H, pst, J = 9.6 Hz, C(3′)H), 7.33–7.37 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 11.6, 18.1, 20.2, 20.3, 20.4, 22.5, 23.1, 26.7, 27.7, 30.6, 30.7, 32.2, 34.5, 34.6, 34.7 (2×), 34.8, 36.8, 38.9, 41.2, 41.9, 49.9, 52.4, 55.4, 65.3, 66.0, 69.3, 70.9, 71.3, 79.2, 97.9, 127.9 (3×), 128.4 (2×), 136.3, 167.5, 168.9, 169.2, 169.4, 173.1.

Benzyl 3α,12α-dihydroxy-5β-cholan-24-oate-3-β-D-glucuronide methyl ester-triacetate (**12**) was obtained in 77% isolated yield as a white solid, mp: 150–155 °C. δ_H (400 MHz, d_6-DMSO) 0.56 (3H, s, C(18)H_3), 0.84 (3H, s, C(19)H_3), 0.89 (3H, d, J = 6 Hz, C(21)H_3), 1.95 (3H, s, OCOCH_3), 1.97 (3H, s, OCOCH_3), 1.99 (3H, s, OCOCH_3), 3.50–3.54 (1H, m, C(3)H), 3.63 (3H, s, CO_2CH_3), 3.76 (1H, s, C(12)H), 4.42 (1H, d, J = 9.9 Hz, C(5′)H), 4.72 (1H, dd, J = 8,0 9.6 Hz, C(2′)H), 4.90–4.97 (2H, m, C(1′)H, C(4′)H), 5.03–5.10 (2H, m, CH_2Ph), 5.31 (1H, pst, J = 9.6 Hz, C(3′)H), 7.32–7.36 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 12.4, 16.8, 20.2, 20.3, 20.4, 22.5, 22.9, 23.4, 25.9, 26.5, 26.7, 27.1, 28.5, 30.7, 32.7, 33.6, 33.8, 34.5, 34.8, 35.5, 41.3, 46.0, 46.2, 47.4, 52.5, 65.3, 69.3, 70.9, 71.0, 71.1, 71.3, 79.2, 98.1, 128.0 (3×), 128.4 (2×), 136.3, 167.5, 169.0, 169.2, 169.5, 173.2.

Benzyl 3α-hydroxy-5β-cholan-24-oate-3-β-D-glucuronide methyl ester-triacetate (**13**) was obtained in 70% isolated yield as a white solid, mp: 138–140 °C. δ_H (400 MHz, d_6-DMSO) 0.57 (3H, s, C(18)H_3), 0.83–0.85 (6H, m, C(19)H_3, C(21)H_3), 1.95 (3H, s, OCOCH_3), 1.97 (3H, s, OCOCH_3), 1.98 (3H, s, OCOCH_3), 3.52–3.55 (1H, m, C(3)H), 3.63 (3H, s, CO_2CH_3), 4.43 (1H, d, J = 9.9 Hz, C(5′)H), 4.72 (1H, dd, J = 8.0, 9.4 Hz, C(2′)H), 4.90–4.97 (2H, m, C(1′)H, C(4′)H), 5.03–5.11 (2H, m, CH_2Ph), 5.31 (1H, pst, J = 9.6 Hz, C(3′)H), 7.31–7.36 (5H, m, C_6H_5). δ_C (100.6 MHz, d_6-DMSO) 11.7, 18.0, 20.2, 20.3, 20.4 (2×), 23.0, 23.7, 25.9, 26.6, 26.7, 27.6, 30.5, 30.6, 33.6, 34.1 (2×), 34.4, 34.7, 35.3, 40.7, 41.3, 42.2, 52.4, 55.3, 55.7, 65.3, 69.3, 70.9, 71.0, 71.3, 79.1, 98.0, 127.9, 128.0 (2×), 128.4 (2×), 136.2, 167.4, 168.9, 169.2, 169.4, 173.1.

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