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Simple method for simultaneous quantification of two new derivatives of betulinic and ursolic acids with antimalarial activity by RP-HPLC-UV-CAD

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Endemic in more than 90 countries and territories, malaria is the most widely, populational, and geographically, parasitic disease in the world. *Plasmodium sp*. resistance to available drugs is one of the biggest problems for malaria eradication. In this study, we develop a method for the simultaneous determination of two new derivatives of betulinic and ursolic acids with antimalarial activity designated 3-O-butanoylbetulinic and 3-O-butanoylursolic acids. An analytical method was developed by high-performance liquid chromatography coupled, in series, to ultraviolet (UV) and charged aerosol (CAD) detectors. The chromatographic system, operated isocratically by reversed-phase, consisted in a mobile phase composed of acetonitrile: water pH 3.0 adjusted with formic acid (85:15, v/v), flow rate of 1.2 mL/min and a PhenoSphere Next octadecylsilane column (250 mm x 4.6 mm, 5 µm particle size). Chromatograms were recorded simultaneously in UV and CAD, at a concentration of 50 µg mL-1, an injection volume of 20 µL at a controlled temperature of 50 °C. The method was found to be selective, linear (r > 0.99), precise (RSD < 2.0%), accurate, and robust for both analytes, and considered statistically validated, and can be applied to the identification and quantification of these new drug candidates.

Keywords: Malaria, HPLC method, charged aerosol detector, drug candidates, mass balance.
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Introduction

Malaria is considered one of the greatest challenges of the 21st century. In 2018, an estimated 228 million cases of malaria occurred worldwide, reported by 91 countries and territories, compared with 231 million cases in 2017. In the same year, an estimated US$ 2.7 billion was invested around the world in malaria control and elimination by governments of malaria endemic countries [1].

The pathology, caused by several protozoa of the genus *Plasmodium sp.*, has vectoral transmission by mosquitoes *Anopheles sp.*, which contribute to the broad geographic distribution and the difficulty of controlling the disease [2]. Furthermore, the resistance of the plasmodium to the current drugs is a major problem in the eradication of malaria. In the 1950s, resistance to chloroquine, the most widely used drug, was detected in South Asia and South America for the first time. In 10 years, the problem was observed in the African continent [3]. Recently, there was a rapid worldwide increase in the resistance of *P. falciparum*, the most virulent species, to the drug [4] with children from 1 to 5 years having the highest incidence and mortality [1].

Betulinic acid (BA) is a naturally occurring pentacyclic lupane-like triterpene distributed in the Kingdom Plantae [5]. Several biological activities for BA and its derivatives have been discovered and studied. These include antimalarial [6,7], anti-HIV [8-10], antineoplastic [11], antibacterial [12,13] and antileishmanicidal [14], among others. Ursolic acid (UA) is a triterpenic secondary metabolite with several known activities, such as antimalarial [15], anticancer [16], anti-inflammatory [17] and antioxidant [18]. In this context, Silva et al. developed a semi-synthesis method to a series of betulinic and ursolic acid analogues aiming to improve the therapeutic activity and reduce toxicity of such molecules. The study investigates carbon-3 esterification, resulting in two non-cytotoxic, short side acyl chain compounds (Fig. 1), designated 3-O-butanoylbetulinic acid (3-OBB) and 3-O-butanoylursolic acid (3-OBU), presenting anti-malarial activity against chloroquine-sensitive *P. falciparum* 3D7 up to five times higher (IC_{50} of 5 ± 0,14 µm and 7 ± 0,15 µm, respectively) than its precursors, BA and UA [7].

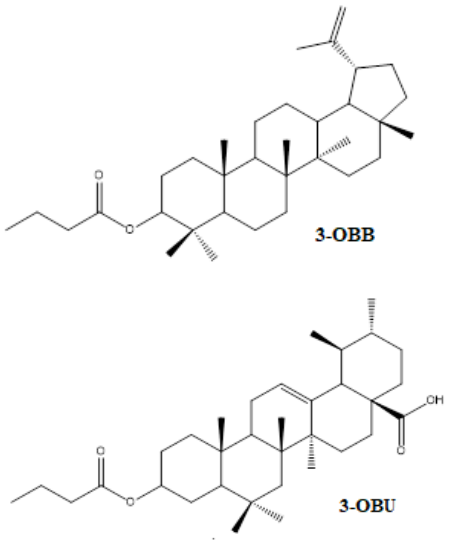

Figure 1. Chemical structures of 3-O-butanoylbetulinic acid and 3-O-butanoylursolic acid.

Quality control of anti-malarial drugs is very important to assure treatment efficacy and to avoid the development of resistance [19]. However, there are no reported methods regarding the identification and quantitation of 3-OBB and 3-OBU. Hence, in this study, a simple and sensitive reversed-phase high-performance liquid chromatography method coupled, in line, to ultraviolet and charged aerosol detectors (RP-HPLC-UV-CAD) for the simultaneous determination of these molecules was developed and validated. Beyond the usual UV detection, charged aerosol detection was chosen because it is considered a universal detector for nonvolatile analytes and has a response independent of chemical properties [20], characteristics that work in favor of low ultraviolet absorptive molecules like 3-OBB and 3-OBU and their impurities. In addition, CAD was used to identify non-chromophore organic impurities in mass balance. The developed method can be applied in further studies with these drug candidates and quality control issues, such as the preparation of chemical reference material that will improve the quality of the data obtained in those studies.

Materials and Methods

Instrumentation

The analytical method was developed and validated in a Shimadzu LC system (Kyoto, Japan) equipped with a LC-20AT gradient pump, DGU-14A vacuum degasser and CTO-10A column oven coupled in series with SPD-M10A Diode Array Detector and Corona CAD (ESA Bioscience, Chelmsford, MA, USA). CAD detection was carried out using nitrogen as nebulizer gas (35 psi). Chromeleon 6.8 software (Dionex Corporation, Sunnyvale, CA, USA) and LC Solutions (Shimadzu, Kyoto, Japan) were used for the instrument control, data acquisition and analysis of the CAD and UV results, respectively.

Chemicals and Materials

BA and UA were provided by the Laboratory of Phytochemistry and Organic Synthesis – Federal University of Rio Grande do Sul, Brazil. BA and UA were obtained from *Platanus acerifolia* bark (2.0% yield) and industrial residue of apple peel *Malus domestica* (2.8% yield), respectively.
Butyric anhydride, dimethylaminopyridine, pyridine and cyclohexane (≥99.0%, ≥99.0%, ≥99.0% and 99.5%) used in the semisynthesis of 3-OBB and 3-OBU acids were obtained from Sigma-Aldrich (Germany). Dichloromethane (99.5%) were purchased from VETEC (Rio de Janeiro, Brazil). Silica gel 60 and silica gel 60 F254 TLC plates used during the purification of the compounds were purchased from Merck (Germany).
Acetonitrile (HPLC grade) and formic acid (98%) were purchased from Merck (Germany). Purified water was produced by a Milli-Q system (Millipore Corp., MA, USA).

Semi-synthesis of 3-OBB and 3-OBU

Semi-synthesis was performed as described by Silva et al [7]. Butyric anhydride (1.1 mmol, 5 Eq), 4-dimethylaminopyridine (0.22 mmol, 1 Eq) and the acid of interest (betulinic or ursolic) diluted in pyridine (0.22 mmol, 1Eq) were added in a round bottom flask and refluxed for 1 h in a nitrogen atmosphere at room temperature. Purification of 3-OBB and 3-OBU were carried out using column chromatography. Silica gel 60 was used as stationary phase and different proportions of dichloromethane and cyclohexane were used as mobile phase. Analytical thin layer chromatography was performed to identify the synthesized compounds [7]. Further purification was performed by recrystallization using 60 °C acetonitrile.
Melting range (FP 90, Mettler Toledo, Switzerland), differential scanning calorimetry (DSC-60, Shimadzu, Japan), infrared spectroscopy (Spectrum BX, Perkin Elmer, USA) and nuclear magnetic resonance spectroscopy (NMR ^{1}H and ^{13}C) (DPX, Bruker, Germany) were used to identify and characterize the synthesized compounds (shown in supplementary data).

Mass balance

For the application of analytical methods, chemical reference standards are commonly used, comparatively, to identity and quantify drug content. In this case, as we synthesized these molecules and there is no compendial standard available, we decided to perform a mass balance to evaluate the purity of the compounds. Mass balance consider inorganic (residue on ignition), organic (HPLC-UV-CAD) and volatile (loss on drying) impurities (100 - impurities%) (n = 3) [21]. Besides quantifying the compounds' purity, mass balance offers the ability to detect shifts in the synthesis and purification processes.

Chromatographic conditions

LC-UV-CAD system was developed using a PhenoSphere Next C18 (250 x 4.6 mm, particle size 5 µm, Phenomenex) column at 50 °C. An isocratic elution was achieved by using a mobile phase consisted of acetonitrile:water with pH adjusted to 3.0 with formic acid (85: 15, v/v). The flow rate was set at 1.2 mL/min and injection volume was 20 µL. For UV detection, a wavelength of 210 nm was used. For CAD detection, nitrogen pressure was set at 35 psi. 3-OBU and 3-OBB were retained in the system for 24 and 27 minutes, respectively.

Standard stock and working solutions

Stock solutions of 3-OBB and 3-OBU were individually prepared by transferring 25.0 mg of each purified substance to a 100 mL volumetric flask. Substances were dissolved in 10 mL of dichloromethane and the volumes were completed with mobile phase to the final concentration of 250.0 µg/mL. The required working

solutions were prepared by further dilutions in mobile phase.

Validation of the analytical method

Method validation was performed according to current guidelines [22, 23].

Selectivity

Selectivity of the method was performed using qualitative individual solutions containing diluent, BA, UA, 3-OBB acid and 3-OBU. A solution containing all compounds, simultaneously, to identify the elution times and to prove the selectivity of the analytical method was also evaluated. BA and UA were chosen, as synthetic precursors, to ensure the method discrimination once they have similar chemical structure to the synthesized 3-OBB and 3-OBU and, therefore, this evaluation was considered critical to validate specificity. Peak purity was verified by UV-DAD detection.

Linearity, LOD and LOQ

The linearity of the method was verified by the construction of three independent calibration curves prepared at five concentration levels: 25.0, 37.5, 50.0, 62.5 and 75.0 µg/mL (50% - 150% of the nominal concentration). Triplicate determinations at each concentration level were performed and concentration versus area values were plotted. Results were statically evaluated by linear regression of the mean curve (α = 0.05), correlation coefficient (r), residue distribution and homoscedasticity. Limit of detection (LOD) and Limit of quantification (LOQ) were determined on the signal-to-noise ratio of 3:1 and 10:1, respectively.

Precision and accuracy

Precision was determined by six replicates of each sample, prepared individually, at the concentration of 50.0 µg/mL, in two different days (day 1 as repeatability and day 2 to accomplish intermediate precision). As there is not a compendial standard available, the ratio between the practical concentration of the samples and the area-response was used as response factor (RF). The relative standard deviation (RSD) of the RF values were evaluated. Accuracy was inferred after the establishment of linearity, selectivity and precision [22].

Solution stability

Solutions of 3-OBU and 3-OBB at working concentrations of 50 µg/mL were tested for 24, 48 and 72 h at 25 ºC. The results were compared with those obtained with the freshly prepared solution.

Results and discussion

HPLC method development

The proposed RP-LC method was optimized with the aim to develop a simple, rapid and suitable analytical method for separation, identification and quantitation of two new 3-O-butanoyl derivatives of betulinic and ursolic acids with anti-malaric activity. Although CAD detection is not as common as UV-DAD, it was a valuable tool in mass balance investigation and might be as valuable in further studies with these two analytes, considering their low molar absorptivity. The developed LC-UV-CAD method demonstrated good selectivity and sensitivity to identify and quantify 3-OBB and 3-OBU in the presence of related molecules (BA and UA).

The final chromatographic conditions established by the authors were selected after the robustness study, evaluating changes such as different batch column, flow rate (1.1 mL/min and 1.3 mL/min), pH of the aqueous phase (2.9 and 3.1) and mobile phase composition (83:17, v/v) and (87:13, v/v). Conditions were chosen based on peak performances (such as theoretical plates, capacity factor, tailing factor) and the best resolution between analytes.

Different chromatographic systems were tested using different columns polarities and sizes. Separation between these similar structured acids, with adequate resolution, was possible with the use of a C_{18} 250 x 4.6 mm, 5.0 µm column. The wavelength was defined by the evaluation of the absorptivity profile of the molecules.

Mass Balance

Recommended by the world health organization (WHO) and official compendia [24], mass balance demonstrated to be an accurate and reproducible methodology to assess the compound purities [25].

After purification, final purities of 94.56% and 83.38% were achieved, for 3-OBB and 3-OBU, respectively. Organic impurities were evaluated in UV and CAD, and the impurities with biggest areas (%) were considered to the mass balance. Chromatographic profile was different in both detectors, with an increased number of impurities detected by CAD, inferring the absence of chromophores in those impurities. Compound 3-OBU acid presented high profile of organic impurities, however, the purity of 83.38% did not interfere with the identification and characterization of the compound, but further purification should be done to establish a reference standard.

Results are shown in table 1.

Table 1. Purity results of 3-OBB and 3-OBU acids achieved by mass balance.

Compound	Inorganic Impurities[a]	Organic Impurities[b]	Volatile impurities[c]	Total impurities	Total purity
3-OBB	0.01±0.0002%	5.10 ± 0.18%	0.32 ± 0.04%	5.43 ± 0.2202%	94.57 ± 0.2202%
3-OBU	0.08 ±0.0009%	15.78 ± 1.21%	0.75 ± 0.06%	16.62 ± 1.9609 %	83.38 ± 1.9609%

[a] Residue on ignition (USP 43); [b] HPLC-UV-CAD (same validated method was applied); [c] Loss on drying (USP 43). Data shown represent the mean of n=3 independent samples.

Method Validation

Selectivity

Selectivity of the method was demonstrated by adequate resolution among BA, UA, 3-OBB and 3-OBU (3.1 between the synthesized compounds). Also, matrix components, impurities and the chromatographic system (e.g. diluent) did not interfere with 3-OBB and 3-OBU peaks (Fig. 2). Peak purity by UV-DAD detection was higher than 0.99 for both peaks.

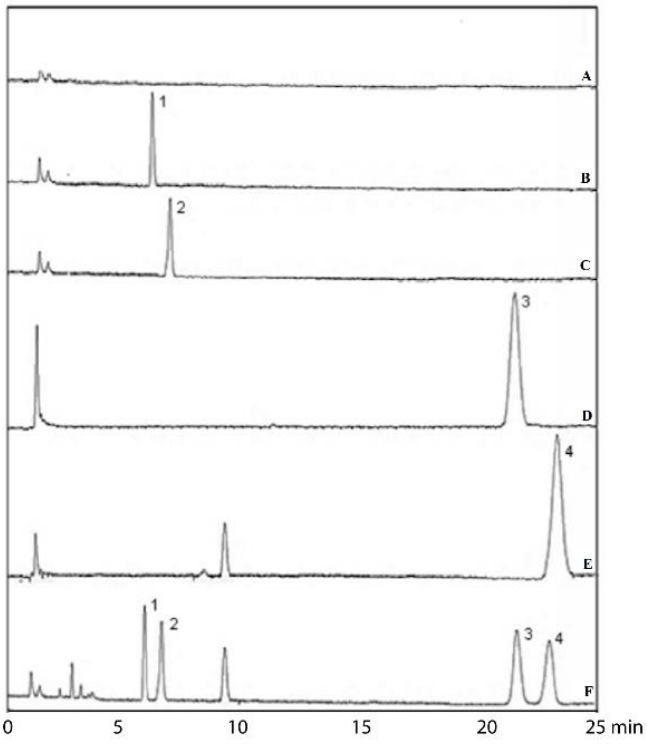

Figura 2. Chromatograms of diluent (A), betulinic acid (B) (peak 1), ursolic acid (C) (peak 2), 3-OBB (D) (peak 3), 3-OBU (E) (peak 4) and a solution containing all compounds (F).

Linearity, LOD and LOQ

All calibration curves were linear in the range of 50 – 150% of nominal concentration (25.0 to 75 µg/mL). The mean correlation coefficient (r) values were > 0.996 for both analytes. Linear regression was applied to confirm the method linearity, and, by residual analysis, it was verified the absence of atypical samples and the homoscedasticity of the residues. LOD was found to be 0.92 µg/mL (UV) and 1.11 µg/mL (CAD) for 3-OBB; 0.92 µg/mL (UV) and 1.16 µg/mL (CAD) for 3-OBU. LOQ was 2.81 µg/mL (UV) and 3.37 µg/mL (CAD) for 3-OBB; 1.29 µg/mL (UV) and 3.50 µg/mL (CAD) for 3-OBU.

Linear regression equations and respective correlation coefficients are presented in table 2.

Table 2. Results of the linearity curves, equations and intercept deviation.

Compound	Detection	Linear equation	σ Y-axis[a]	Correlation Coefficient (r^2)
3-OBB	UV	6009.8x - 20467	0.26	0.9978
	CAD	0.2496x + 0.1786	0.34	0.9983
3-OBU	UV	3975.3x – 4654.7	0.12	0.9981
	CAD	0.1355x – 0.3864	0.32	0.9958

[a] Standard deviation of the Y-axis intercept (n = 3).

Precision and accuracy

Experimental data obtained from the repeatability and intermediate precision are shown in table 3. Values of intra and interday RSD were below 2.0% for 3-OBB and 3-OBU in both UV and CAD.

Table 3. Results of repeatability and intermediate precision of 3-OBB and 3-OBU acids by UV and CAD.

Compound	UV		CAD	
	Day 1	Day 2	Day 1	Day 2
3-OBB				
Repeatability (µg/mL)[a]	50.0	50.5	50.0	50.5
RF (µg/mL/A)[b]	5502.30	5563.30	0.2553	0.2553
RSD %[c]	1.02	0.70	1.25	1.43
Intermediate precision (µg/mL)[d]		50.2		50.2
RF (µg/mL/A)		5532.80		0.2553
RSD %		1.01		1.28
3-OBU				
Repeatability (µg/mL)[a]	47.8	49.9	47.8	49.9
RF (µg/mL/A)[b]	3494.39	3491.80	0.1291	0.1285
RSD %[c]	1.07	1.10	1.13	1.11
Intermediate precision (µg/mL)[d]		48.9		48.9
RF (µg/mL/A)		3493.09		0.1288
RSD %		1.04		1.10

[a] Mean concentration of six replicates, [b] response factor; [c] relative standard deviation (%); [d] mean concentration of twelve replicates.

As stated previously, in accordance with ICH, since there is no SQR available, accuracy was inferred after the establishment of linearity, selectivity and precision.

Solution Stability

The stability of 50.0 µg/mL solutions of 3-OBU and 3-OBB acids were tested for 24, 48 and 72 h at room temperature. High stability (RSD < 2.0% in comparison with fresh solution) was observed under the tested condition in UV and CAD.

Conclusion

The analytical method developed for the simultaneous analysis of antimalarials 3-O-butanoylbetulinic and 3-O-butanoylursolic acids by RP-HPLC-UV-CAD demonstrated to be suitable for its purpose. This simple, robust and sensitive technique can be applied with reliability to identify and quantify these drug candidates in further studies.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

Supplementary Data

Melting range (FP 90, Mettler Toledo, Switzerland)

Table 4. Melting range results for 3-OBB and 3-OBU compounds.

Sample	Melting range	Melting range (n = 3)
3-OBB	313.5 °C – 350.8 °C	312.9 °C – 351.0 °C
	312.8 °C – 348.8 °C	
	312.3 °C – 353.3 °C	
3-OBU	255.9 °C – 284.5 °C	256.3 °C – 283.2 °C
	257.8 °C – 281.4 °C	
	255.3 °C – 283.8 °C	

Differential scanning calorimetry (DSC-60, Shimadzu, Japan)

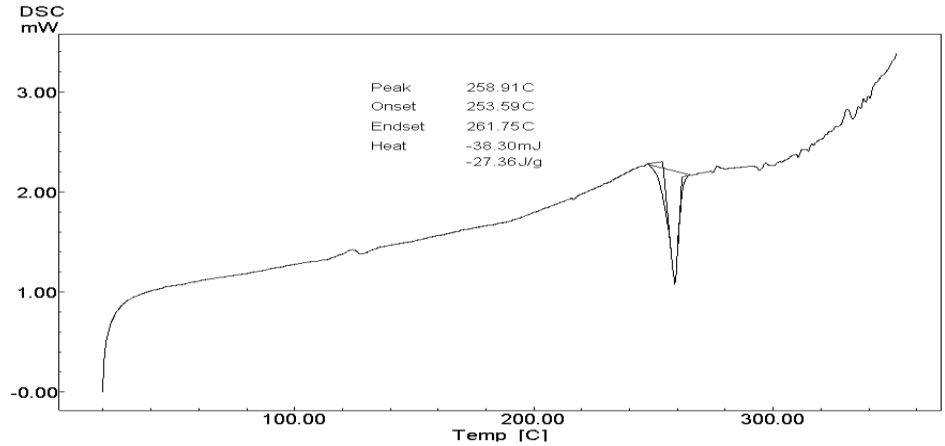

Figure 3. Heating curve of 3-OBU obtained by DSC (10 °C/min).

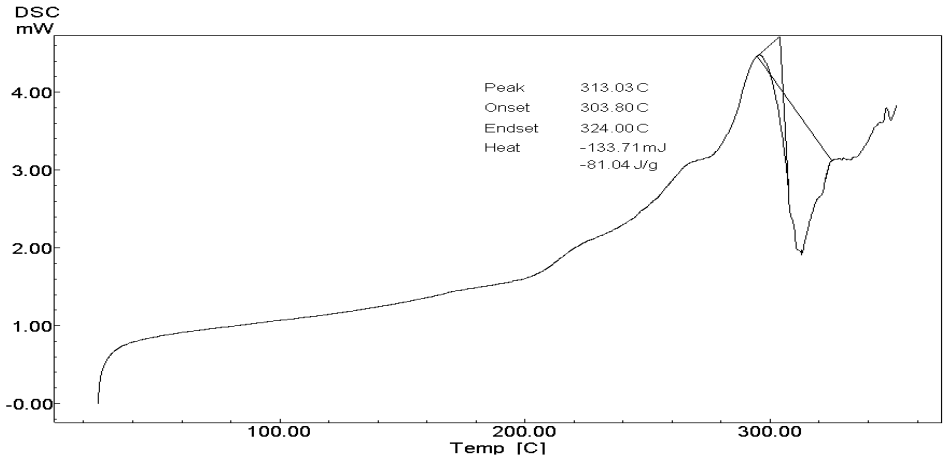

Figure 4. Heating curve of 3-OBB obtained by DSC (10 °C/min).

Nuclear magnetic resonance spectroscopy (NMR 1H and 13C) (DPX, Bruker, Germany)

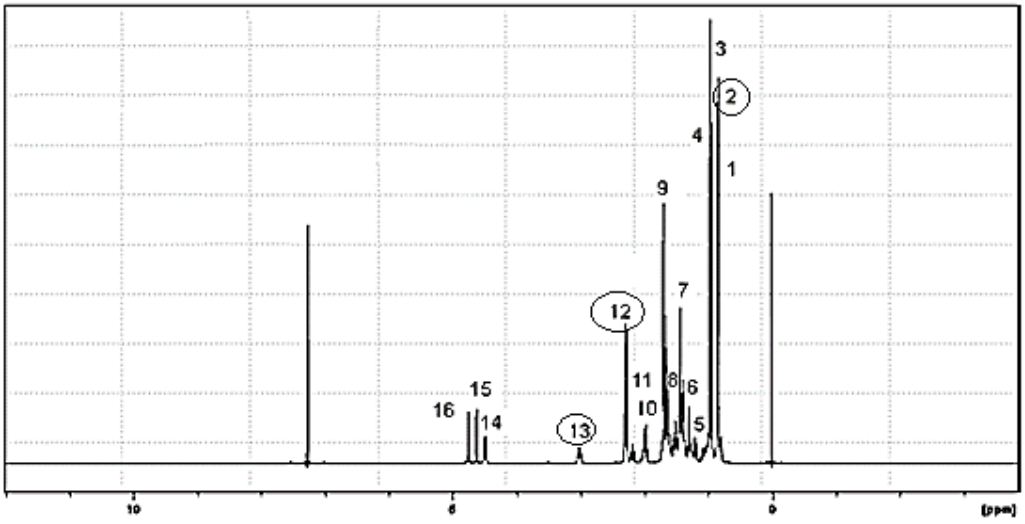

Figure 5. RMN ^{1}H of 3-OBB (400 MHz, CDCl3). Hydrogens highlighted refer to the ones substituted in 3C.

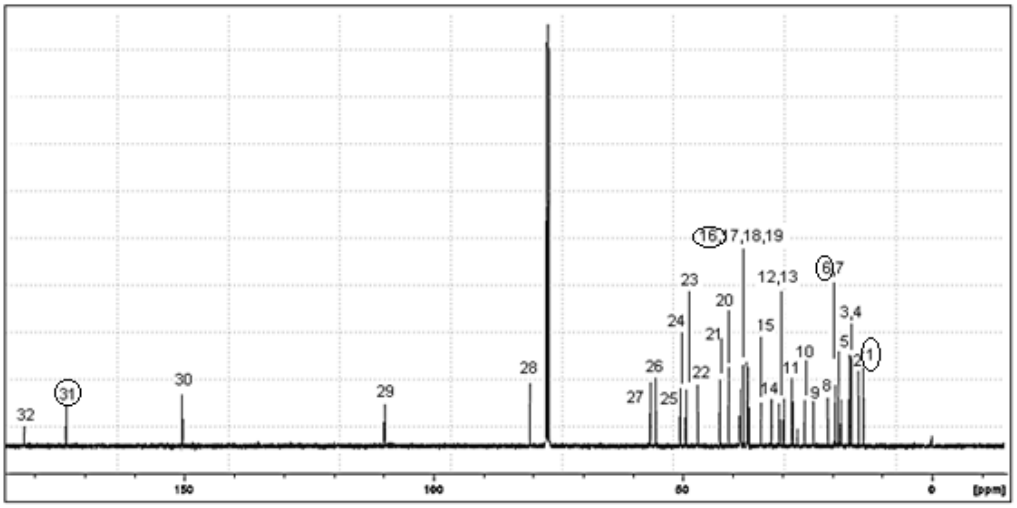

Figure 6. RMN ^{13}C of 3-OBB (400 MHz, CDCl3). Carbons highlighted refer to the ones substituted in 3C.

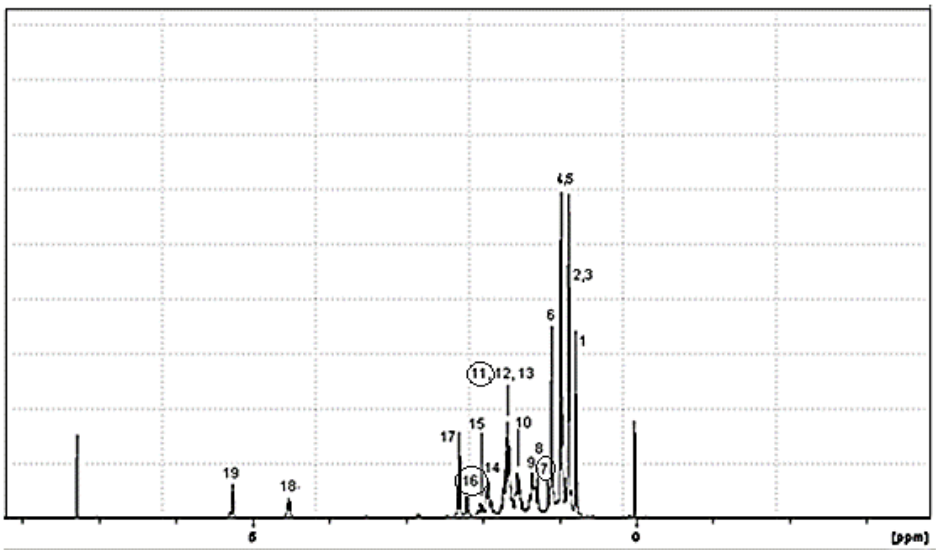

Figure 7. RMN ^{1}H of 3-OBU (400 MHz, CDCl3). Hydrogens highlighted refer to the ones substituted in 3C.

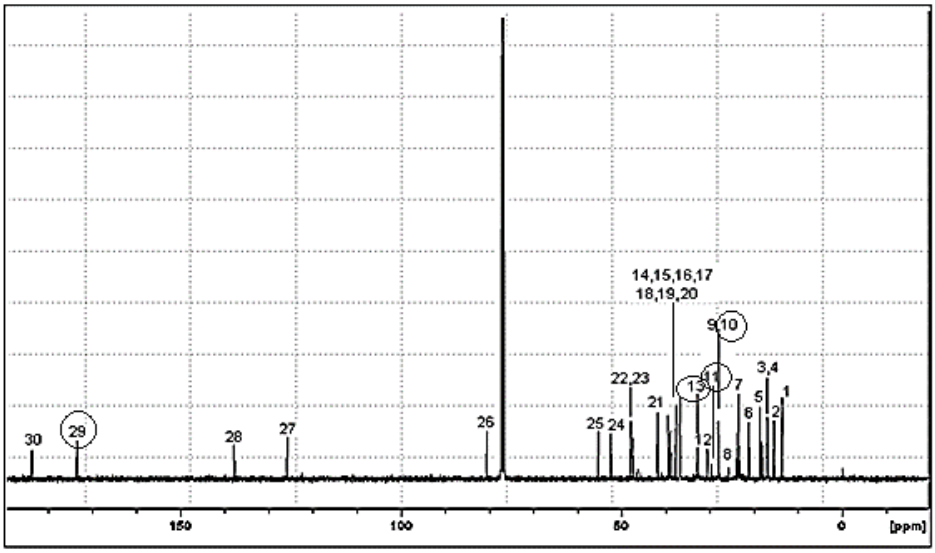

Figure 8. RMN ^{13}C of 3-OBU (400 MHz, CDCl3). Carbons highlighted refer to the ones substituted in 3C.

Table 5. RMN 1H of 3-OBB attributions.

Position	δ (ppm)	Multiplicity – Number of hydrogens	Attribution
1	0.90	s – 3H	CH3
2	0.96	s – 3H	CH3
3	0.98	s – 6H	CH3; CH3
4	1.04	s – 3H	CH3
5	1.45	m – 2H	CH2
6	1.50	m – 1H	CH
7	1.62	m – 2H	CH2
8	1.76	t – 1H	CH
9	1.80	s – 3H	CH3
10	2.04	m - 2H	CH2
11	2.24	t – 2H	CH2
12	2.31	m – 6H	CH2; CH2; CH2
13	3.14	t - 2H	CH2
14	4.53	dd – 1H	CH
15	4.64	s – 1H	CH
16	4.76	s – 1H	CH

Table 6. RMN ^{13}C 1H of 3-OBB attributions.

Position	δ (ppm)	Attribution
1	13.0	C 34
2	15,0	C 27
3	16.0	C 26
4	16.5	C 25
5	18.1	C 6
6	18.6	C 33
7	19.3	C 30
8	20.8	C 11
9	23.6	C 2
10	25.6	C 12
11	27.9	C 23; C 24
12	29.7	C 15
13	30.6	C 21
14	32.1	C 16
15	34.2	C 7
16	36.7	C 22; C 32
17	37	C 10
18	37.1	C 4
19	37.8	C 13
20	38.4	C 1
21	40.7	C 8
22	42.4	C 14
23	46.9	C 19
24	49.3	C 18
25	50.4	C 9
26	55.4	C 17
27	56.4	C 5
28	80.6	C 3
29	109.7	C 20
30	150.3	C 29
31	173.5	C 31
32	183.5	C 28

Table 7. RMN 1H of 3-OBU attributions.

Position	δ (ppm)	Multiplicity – Number of hydrogens	Attribution
1	0.70	s – 3H	CH3
2	0.84	s – 6H	CH3
3	0.87	s – 3H	CH3
4	0.92	s – 3H	CH3
5	1.00	s – 3H	CH3
6	1.05	d – 3H	CH3
7	1.15	t – 3H	CH3
8	1.19	s – 3H	CH3
9	1.25	d – 3H	CH3
10	1.52	m - H	CH
11	1.73	m – 2H	CH2
12	1.77	m – 2H	CH2
13	1.80	m - H	CH2
14	1.96	dd – 2H	CH2
15	2.08	d - H	CH
16	2.23	t – 2H	CH2
17	2.39	s – 1H	CH
18	4.55	dd – 1H	CH
19	5.28	s – 1H	CH

Table 8. RMN ^{13}C 1H of 3-OBU attributions.

Position	δ (ppm)	Attribution
1	14.0	C 25
2	15.3	C 27
3	16.6	C 26
4	16.9	C 29
5	18.1	C 6
6	21.6	C 30
7	23.1	C 23; C 24
8	25.8	C 11
9	27.6	C 2
10	27.7	C 16; C 33
11	29.8	C 15
12	31.5	C 21
13	33.0	C 7; C 32; C 34
14	33.8	C 22
15	36.9	C 10
16	37.7	C 4
17	38.2	C 1
18	38.8	C 19
19	39	C 20
20	39.5	C 8
21	41.9	C 14
22	47.3	C 9
23	47.9	C 17
24	52.5	C 18
25	55.3	C 5
26	80.5	C 3
27	125.7	C 12
28	138.4	C 13
29	173.5	C 31
30	184.0	C 28

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