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From Retrospective Assessment to Prospective Decisions in Natural Product Isolation: HPLC-SPE-NMR Analysis of *Carthamus oxyacantha*

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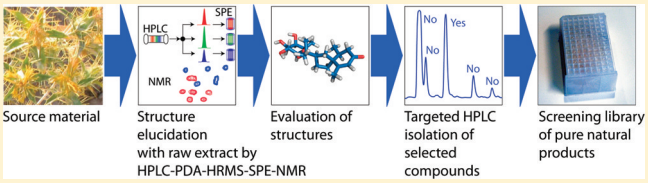
Supporting Information

ABSTRACT: An extract of *Carthamus oxyacantha* (wild safflower) was investigated using two approaches: a traditional, nontarget fractionation by VLC and HPLC, and the hyphenated technique HPLC-PDA-HRMS-SPE-NMR followed by targeted isolation of selected constituents for inclusion in a screening library of pure natural products. While the nontarget fractionation involved considerable time spent on pursuing fractions containing well-known or undesired compounds, the hyphenated analysis was considerably faster and required less solvent and other consumables. The results were used to design and execute an optimized, HPLC-HRMS-guided, targeted isolation scheme aiming exclusively at a series of identified spiro compounds. Thus, HPLC-PDA-HRMS-SPE-NMR is a dereplication technique of choice, allowing economical acquisition of comprehensive data about compounds in crude extracts, which can be used for rational, prospective decisions about further isolation efforts. A total of 15 compounds were identified in the extract. Six spiro compounds, of which four have not previously been characterized, and tracheloside (a lignin glucoside) are presented with assigned ^{1}H and ^{13}C chemical shifts.



Repetitive isolation of known or even readily available natural products is one of the main factors limiting productivity of natural products research.[1−3] Dereplication, i.e., recognition and exclusion from further isolation efforts of extract constituents that have already been studied or are otherwise unwanted, is therefore a cornerstone of lead discovery programs based on natural products.[4−6] A number of techniques, mainly HPLC with mass spectrometric (HPLC-MS) or photodiode array (HPLC-PDA) detection, supported by databases are being used for early recognition of known or unwanted chemical entities in extracts.[7−12] However, natural products are best identified by NMR spectroscopy at a homogeneous stage, which normally requires a laborious fractionation and purification process. In summary, the assessment of the value of isolation efforts undertaken is traditionally retrospective in nature and as such cannot be used to make early stop-or-go decisions while the fractionation is in progress.

Advances in miniaturization of NMR probes have enabled full or partial structure elucidation of extract components following microfractionation in 96-well plate format in parallel with bioassays.[13,14] This approach not only allows elimination of known and unwanted compounds but can led to full structure elucidation of new natural products and therefore assessment of their value at an early stage. Another dereplication approach, having the advantage of rendering the evaporation of HPLC fractions unnecessary and conducting the whole operation in an automated fashion, is the use of HPLC-SPE-NMR hyphenation.[15−18] In this technique, analytes are removed from the HPLC mobile phase by solid-phase extraction (SPE) to be subsequently eluted from the SPE column with a deuterated solvent for NMR analysis. It has been demonstrated that this approach can provide largely comprehensive information about chemical composition of crude extracts, thus enabling early and prospective assessment of the value of an extract or fraction, including decisions about which peaks in a HPLC chromatogram represent valuable components to be isolated, e.g., for pharmacological screening. In this article, we describe a study of an extract of *Carthamus oxyacantha* using two approaches. In the first, classical approach as many extract components as possible were isolated by preparative HPLC for final identification by spectroscopic methods at a purified stage. In the second approach, the extract was first analyzed by HPLC-PDA-HRMS-SPE-NMR, and selected components were fully identified. These components were subsequently purified using a targeted isolation procedure. The outcome of these two approaches, including investment in time and materials, is compared.

The genus *Carthamus*[19] (Asteraceae) comprises herbaceous, thistle-like plants distributed in western and central Asia as well as in the Mediterranean region. *Carthamus tinctorius* L. (safflower), cultivated mainly for its oil-rich seeds, is the best known representative of the genus. *Carthamus oxyacantha* M. Bieb. (wild safflower) is also known for its fatty acid content,[20−22] but otherwise the knowledge of its chemistry is limited. Various glycosides[23] and sesquiterpenoids,[24] including an unusual derivative of spiro[4.5]decane (**1**),[25] were isolated from *C. oxyacantha*. Crude extracts of this plant were

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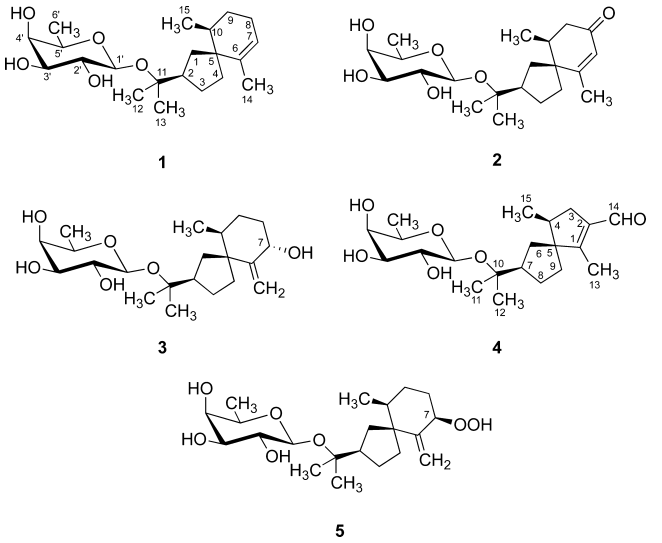
investigated for cholinergic and calcium channel blocking activities[26] and cholinesterase-inhibitory activity.[27]

RESULTS AND DISCUSSION

The traditional fractionation of the crude ethanol extract of aerial parts of *C. oxyacantha*, aimed at isolation of as many pure compounds as possible, was initiated with vacuum liquid chromatography (VLC) to give four fractions, further purified by preparative HPLC with UV detection. This resulted in isolation of vanillic acid, the known lignan glucoside tracheloside,[28] four spiro compounds[25,29] **1**–**4**, (2*Z*,4*R*)-hydroxynon-2-enoic acid,[30,31] and 4-(β-D-glucopyranosyloxy)-3-methoxybenzoic acid.[32] In addition, several fractions were obtained that contained saturated fatty components, but these fractions were not investigated further after recording their ^{1}H NMR spectra. Furthermore, a number of fractions contained caffeic acid derivatives and flavonol and chalcone glycosides (^{1}H NMR), but the sites of *O*-glycosidation and *O*-methylation and the nature of sugar residues were not studied. The spirane **1**, hinesol β-D-fucopyranoside, was previously isolated from *C. oxyacantha*,[25] whereas **3** was reported from a related species, *Carduncellus mareoticus*.[29] In summary, the preparative scale fractionation yielded four interesting spiro compounds, two of which are new (compounds **2** and **4**), but also several nuisance compounds belonging to common types of natural products (fats, flavonoids, and other polyphenols). However, the general chemical nature of these compounds was disclosed only after considerable investment in fractionation and fraction evaporation efforts, which is a crucial weakness of this nontargeted approach.



For HPLC-PDA-HRMS-SPE-NMR analysis, the crude extract was prepurified by partitioning between CH_3OH–H_2O (9:1) and petroleum ether to remove the bulk of chlorophyll and hydrophobic constituents such as fats, fatty acids, and waxes. HPLC traces of the defatted extract are shown in Figure 1. In contrast to earlier studies at this laboratory where a flow NMR probe has been used,[15−17,33,34] the hyphenated system used in this work employed 1.7 mm tubes and the NMR spectra were acquired with a cryogenically cooled probe. Because elution of the SPE cartridges into the capillary NMR tubes takes place with a robotic liquid handler and the tubes are managed by an automated sample changer,

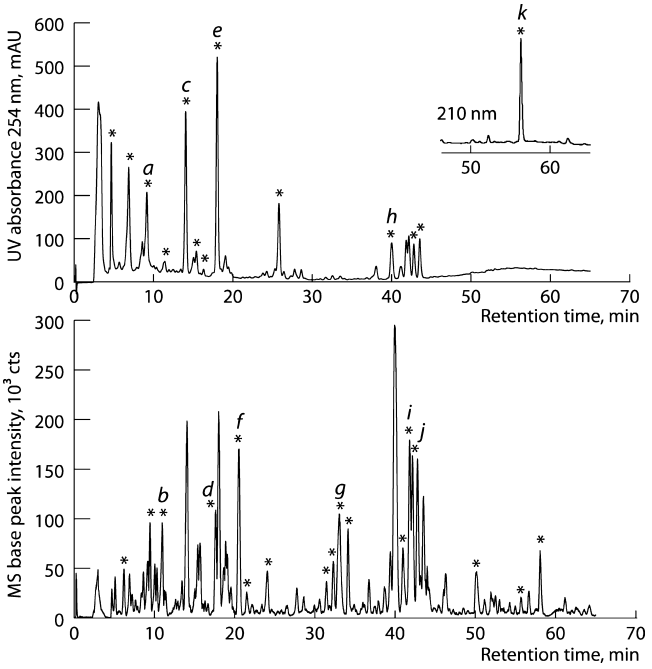


Figure 1. HPLC traces of UV absorbance at 254 nm (top) and MS base peak chromatogram (bottom) of defatted extract of aerial parts of *C. oxyacantha*. Asterisks indicate 30 peaks selected for initial analysis. Peaks *a*–*k* were selected for multiple trapping for further analysis, whereas the remaining peaks, apparently corresponding to phenolic and fatty constituents (^{1}H NMR), were not further analyzed. Trapping was performed using thresholds of 254 nm absorbance or thresholds of MS signals as shown (except for peak *k*, which was trapped by using the 210 nm signal). HPLC conditions: Phenomenex Luna C18(2) column, 4.6 × 150 mm, 5 μm, 25 °C, MeCN gradient in H_2O (each solvent contaning 5% of the other solvent and 1% of HCOOH) from 20% to 100% in 50 min at 0.5 mL/min.

both requiring a minimal participation of an operator, and because the cryogenically cooled microprobe offers a considerably increased sensitivity,[35,36] this system is regarded as an improved and more advantageous implementation of hyphenation of HPLC with NMR spectroscopy. Moreover, a hybrid quadrupole TOF mass spectrometer integrated in the system was capable of providing molecular formulas by combination of HR mass detection and isotope peak examination software. For the initial analysis, 30 peaks (Figure 1) were selected for adsorption on SPE cartridges to obtain 1D ^{1}H NMR spectra. For each peak two or three cumulative trappings were performed based on UV or MS thresholds, respectively. Most of the peaks seen in the chromatograms (Figure 1) were shown by ^{1}H NMR data to correspond to saturated fatty acids and flavonoids. On the basis of these initial ^{1}H NMR data, 11 peaks (peaks *a*–*k*, Figure 1) were selected for eight cumulative trappings for additional studies by 2D NMR. Several peaks (*h*–*k*) exhibited ^{1}H NMR characteristics of fatty acids (the presence of a distorted triplet corresponding to a terminal methyl group at about δ_H 0.90, a methylene envelope around δ_H 1.30, and an α-methylene group triplet at δ_H 2.25), but were nevertheless selected for the more detailed analysis because of the presence of noteworthy olefinic proton patterns.

The compound eluted as peak *a* (t_R = 9.8 min, *m*/*z* 551.2121, $[M + H]^+$ corresponding to $C_{27}H_{35}O_{12}^+$, ΔM 0.4 ppm) was the known tracheloside,[28] a lignan glucoside previously isolated[37] from *C. tinctorius* and also common in

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Table 1. NMR Data for Compounds 1−5 Acquired in the HPLC-SPE-NMR Mode[a]

	1		2		3		4		5	
position	δ_C	δ_H (J in Hz)	δ_{C44}	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	33.9	1.44, dd (13.3, 12.2)	32.0	1.55, dd (13.3, 12.0)	33.4	1.29, dd (13.2, 9.8)	169.9		34.1	1.49, m
		1.62, m		1.94, m		1.82, m				1.74, m
2	51.9	2.05, m	51.6	2.14, m	52.0	2.02, m	137.0		49.7	2.06, m
3	28.6	1.58, m	28.4	1.71, m	27.4	1.52, m	36.1	1.93, m	26.5	1.58, m
		1.72, m		1.80, m		1.63, m		2.50, m		1.65, m
4	36.3	1.58, m	35.4	1.80, m	34.1	1.72, ddd (13.0, 11.4, 6.8)	42.5	1.94, m	35.5	1.74, m
		1.64, m				1.93, m				1.84, m
5	49.4		51.3		52.6		62.9		52.6	
6	141.6		169.4		159.4		30.9	1.32, dd (13.2, 12.0)	152.9	
								1.83, m		
7	121.9	5.28, m (2H)	125.7	5.66, br s	71.2	4.07, br m[c]	51.5	2.07, m	86.3	4.35, dd (6.7, 4.2)
8	24.9	1.92, m	199.9		36.7	1.21, m	27.8	1.61, m	29.7	1.51, m
						1.91, m		1.75, m		1.91, m
9	28.8	1.33, m	43.3	2.16[b]	31.0	1.35, m	35.6	1.47, dt (12.9, 8.2)	28.2	1.29, m
		1.58, m		2.34, dd (16.6, 4.1)		1.64, m		1.81, m		1.85, m
10	37.8	1.54, m	37.9	2.07, m	40.6	1.36, m	78.9		37.9	1.58, m
11	79.1		78.7		78.9		24.7	1.20, s	79.0	
12	24.6	1.18, s	24.6	1.21, s	24.3	1.11, s	25.6	1.21, s	24.4	1.15, s
13	25.4	1.19, s	25.9	1.22, s	25.5	1.16, s	10.8	2.08, s	25.7	1.18, s
14	20.1	1.68, td (2.0, 1.5)	20.9	1.97, m	101.5	4.81, br s	190.0	9.96, s	108.5	4.95, t (1.4)
						4.98, t (1.6)				4.96, t (1.2)
15	16.6	0.93, d (6.9)	16.6	1.00, d (6.6)	16.7	0.88, d (6.5)	14.8	0.99, d (6.7)	15.7	0.86, d (6.8)
1′	98.0	4.34, d (7.7)	98.0	4.35, d (7.7)	97.9	4.31, d (7.7)	98.2	4.35, d (7.6)	98.2	4.33, d (7.7)
2′	72.4	3.21, m	72.3	3.23, m	72.3	3.20, m	72.5	3.22, m	72.5	3.23, m
3′	74.6	3.42, m	74.5	3.41, m	74.4	3.39, m	74.7	3.41, m	74.6	3.40, m
4′	72.3	3.52, m	72.2	3.53, m	72.2	3.52, m	72.4	3.53, m	72.3	3.53, m
5′	70.6	3.56, qd (6.5, 1.2)	70.5	3.57, qd (6.5, 1.2)	70.5	3.55, qd (6.5, 1.2)	70.7	3.56, qd (6.5, 1.2)	70.6	3.56, qd (6.5, 1.2)
6′	16.9	1.17, d (6.5)	16.9	1.17, d (6.5)	16.9	1.17, d (6.5)	16.9	1.16, d (6.5)	16.9	1.17, d (6.5)

[a]600 MHz spectra in acetonitrile-d_3; d, doublet; t, triplet; q, quartet, m, multiplet, br, broad signal; ^{13}C chemical shifts from one- and multiple-bond proton-detected 2D heteronuclear correlations. [b]Overlapped by water signal, ^{1}H NMR spectrum of the isolated compound in methanol-d_4 exhibited dd (16.6, 10.0) at δ_H 2.17. [c]In the hyphenation-mode spectrum in methanol-d_4, the signal appears as ddt (10.4, 4.6, 1.3).

the genus *Trachelospernum* (Apocynaceae).[38,39] Since complete ^{1}H and ^{13}C NMR data for tracheloside have apparently not been reported, they are included in the Supporting Information (Table S1). The compound eluted as peak *b* (t_R = 10.7 min, *m/z* 189.1119, $[M + H]^+$ corresponding to $C_9H_{17}O_4^+$, ΔM 1.3 ppm) was azelaic acid (^{1}H NMR, COSY, NOESY, HMBC, HSQC).

The compound eluted as peak *c* (t_R = 13.9 min, *m/z* 383.2426, $[M + H]^+$ corresponding to $C_{21}H_{35}O_6^+$, ΔM 0.6 ppm) was the spiroketone **2**. By comparison of its ^{13}C chemical shifts (obtained through HSQC and HMBC spectra) to those of hinesol-β-D-fucopyranoside (**1**), the compound was found to have the same sugar residue and the same spirocyclic skeleton as the latter. Analysis of 2D NMR data allowed assignment of all ^{1}H and ^{13}C resonances of **2** (Table 1). From the coupling constants between H-10 and the protons H-9a and H-9b ($^3J_{9a,10}$ = 10.0 Hz, $^3J_{9b,10}$ = 4.1 Hz), it can be concluded that H-10 is pseudoaxial, whereas the methyl group is pseudoequatorial. Accordingly, H-10 displayed a NOESY correlation to H-4, whereas the C-15 methyl protons displayed correlations to H-1, H-4, and H-2, in agreement with the relative configuration of the sesquiterpene portion of the molecule. The spiroketone is new, although related compounds have previously been isolated from tobacco and fungus-infected potatoes.[40−42]

The compound eluted as peak *d* (t_R = 17.4 min, *m/z* 385.2579, $[M + H]^+$ corresponding to $C_{21}H_{37}O_6^+$, ΔM 1.5 ppm) was identified as the known spiroalcohol **3**.[29] Two epimeric alcohols have previously been described,[29] differing by the coupling pattern of H-7. One exhibited H-7 as a double doublet with $^3J_{7,8}$ = 4 and 11 Hz, and the other with $^3J_{7,8}$ = 3.5 and 7.5 Hz.[29] The multiplicity of the H-7 signal in the hyphenation-mode ^{1}H NMR spectrum acquired in acetonitrile-d_3 was obscured by partial coupling to the hydroxylic proton due to slow chemical exchange. However, a hyphenation-mode spectrum in methanol-d_4 showed $^3J_{7,8}$ = 4.6 and 10.4 Hz, the same as the material isolated in the nontarget preparative procedure. This evidently corresponds to the former of the previously reported epimers, as also shown by close similarity of ^{13}C NMR chemical shifts. The coupling constants correspond to the hydroxy group in the equatorial orientation with H-7 axial, and thus the structure **3** is assigned to the compound eluted as peak *d*.

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By HRMS analysis, the compound eluted as peak *e* (t_R = 17.7 min) was assigned the molecular formula $C_{21}H_{34}O_6$ (*m/z* 383.2424, $[M + H]^+$ corresponding to $C_{21}H_{35}O_6^+$, ΔM 1.1 ppm). It exhibited sugar resonances identical to those of **1**−**3** and aglycone resonances comprising two geminal methyl groups (singlets at δ_H 1.20 and 1.21, δ_C 24.7 and 25.6), the C-15 methyl group (doublet at δ_H 0.99, ^3J = 6.7 Hz, δ_C 14.8), and an allylic methyl group [δ_H 2.08 (broad singlet), δ_C 10.8]. In addition, formyl resonances were observed (δ_H 9.90, δ_C 190.0). This relatively low ^{13}C NMR chemical shift indicated conjugation with a double bond. Finally, analysis of HMBC connectivities (Figure 2) established for this compound a new

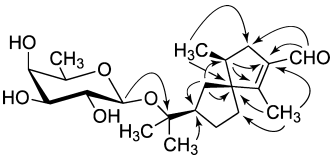


Figure 2. Selected HMBC correlations (from H to C) observed for compound **4** (HMBC spectrum optimized for $^nJ_{C,H}$ = 8 Hz acquired in the hyphenation mode; 600 MHz, acetonitrile-d_3).

spiro[4.4]nonane, **4**, representing a new variation of the sesquiterpene aglycone skeleton of the fucopyranosides produced by this plant.

The compound eluted as peak *f* (t_R = 20.2 min) exhibited general NMR characteristics similar to that of compound **3**, but its mass spectrum demonstrated the presence of an additional oxygen atom (*m/z* 401.2536, $[M + H]^+$ corresponding to $C_{21}H_{37}O_7^+$, ΔM 0.5 ppm). However, analysis of a multiplicity-edited HSQC spectrum and chemical shift comparison showed no evidence of an additional oxygenated carbon atom, while a downfield shift of C-7 (15 ppm) as well as minor upfield shifts for C-5, C-8, and C-9 were observed. These shifts are consistent with the presence of a hydroperoxy group instead of the hydroxy group at C-7.[43,44] Moreover, the coupling constants of the double doublet of H-7 ($^3J_{7,8}$ = 4.2 and 6.7 Hz in acetonitrile-d_3, $^3J_{7,8}$ = 3.1 and 6.4 Hz in methanol-d_4) were different from those of **3** and similar to its known C-7 epimer,[29] indicating an axial orientation of the hydroperoxy group. Thus, in contrast to compound **3**, with the hydroxy group *trans* to the methyl group in the cyclohexane ring, which strongly favors diequatorial conformation, the corresponding substituents in the cyclohexane ring of the hydroperoxide are *cis*, which necessitates axial−equatorial orientation of the substituents. On the basis of these data, the structure **5** is assigned to the compound eluted as peak *e*. Natural products with hydroperoxide structures are rare, but have occasionally been isolated from various sources, notably allylic hydroperoxides as in the present case.[44−48]

The compound eluted as peak *g* (t_R = 32.7 min), for which HRMS afforded the molecular formula $C_{21}H_{36}O_5$ (*m/z* 369.2642, $[M + H]^+$ corresponding to $C_{21}H_{37}O_5^+$, ΔM 1.8 ppm), was the previously mentioned hinesol *β*-D-fucopyranoside (**1**).[25] The structure was confirmed by analysis of COSY, HSQC, and HMBC spectra. A summary of ^1H and ^{13}C NMR data of the five spiro compounds (**1**−**5**) is given in Table 1.

The compounds eluted as peaks *h*, *i*, *j*, and *k* were unsaturated fatty acids. 1D and 2D NMR data, together with the molecular formula $C_{18}H_{32}O_3$ inferred from HRMS (weak peak at *m/z* 319.2237, $[M + Na]^+$ corresponding to $C_{18}H_{32}O_3Na^+$, ΔM 2.1 ppm; base peak at *m/z* 279.2318, $[M - H_2O + H]^+$ corresponding to $C_{18}H_{31}O_2^+$, ΔM 0.2 ppm), strongly indicated that the compound eluted as peak *h* (t_R = 39.7 min) is either (9*Z*,11*E*)-13-hydroxyoctadeca-9,11-dienoic acid (coriolic acid) or (10*E*,12Z)-9-hydroxyoctadeca-10,12-dienoic acid (*α*-dimorphecolic acid). Although the base peak displayed in positive-mode MS corresponded to the formula $C_{18}H_{30}O_2$ rather than $C_{18}H_{32}O_3$ and the second largest peak was $[2 M - 2H_2O + H]^+$, with only weak $[M + H]^+$ and $[M + Na]^+$ ions present, the possibility that the compound is a macrocyclic lactone[49] could be excluded by the chemical shift of the oxygenated methine group (δ_H 4.05), inconsistent with an *O*-acylated hydroxy group. The observed ^1H NMR signals of a CH_2−CH=CH−CH=CH−CH(OH)−CH_2 spin system, with coupling constants corresponding to *Z*- and *E*-double bonds, respectively [δ_H 1.55 (q, $J \approx 7.1$ Hz), δ_C 25.5; δ_H 2.16 (qm, $J \approx 7.4$ Hz), δ_C 28.1; δ_H 4.05 (q, $J \approx 6.5$ Hz), δ_C 72.3; δ_H 5.41 (m), δ_C 132.6; δ_H 5.64 (ddd, J = 15.2, 6.4, and 2.5 Hz), δ_C 138.0; δ_H 5.97 (t, J = 10.9 Hz), δ_C 128.9; δ_H 6.47 (dddt, J = 15.2, 10.9, 2.2 Hz, and 1.0 Hz), δ_C 125.3], together with signals of a CH_2−COOH group [δ_H 2.25 (t, J = 7.4 Hz) correlated to δ_C 34.1 and 173.9], were compatible with either of the isomers mentioned above.[50,51] Nevertheless, the distinction could be made using negative-ion mode MS/MS. Thus, it is well established that the $[M - H]^-$ ion of 13-hydroxyoctadeca-9,11-dienoic acid fragments with formation of a prominent ion at *m/z* 195, with weaker peaks at *m/z* 179 and 113 and no fragments at *m/z* 171 and 123, which in turn are characteristic of 9-hydroxyoctadeca-10,12-dienoic acid.[52−54,56−58] The compound eluted as peak *h* gave a pseudomolecular ion at *m/z* 295.2276 ($[M - H]^-$ corresponding to $C_{18}H_{31}O_3^-$, ΔM 1.0 ppm), with a prominent fragment at *m/z* 171.1023 ($[M - CH_3(CH_2)_4CHCHCHCH_2 - H]^-$, ΔM 2.4 ppm), and a weaker fragment at *m/z* 123.1181 ($[M - HOOC(CH_2)_7CHO - H]^-$, ΔM 1.7 ppm), both originating from scission of the C-9−C-10 bond. This excludes the 13-hydroxy isomer and proves the compound eluted as peak *h* to be (10*E*,12Z)-9-hydroxyoctadeca-10,12-dienoic acid. The absolute configuration was not determined.

The compounds eluted as peaks *i* and *j* (t_R = 41.5 and 41.9 min) gave similar HRMS data, with *m/z* 317.2079 and 317.2076, respectively ($[M + Na]^+$ corresponding to $C_{18}H_{30}O_3Na^+$, ΔM 2.6 and 3.4 ppm, respectively), and *m/z* 295.2260 and 295.2260, respectively ($[M + H]^+$ corresponding to $C_{18}H_{31}O_3^+$, ΔM 2.5 and 2.5 ppm, respectively). 1D and 2D NMR data acquired in the hyphenation mode were compatible with the structure of (9*Z*,11*E*)-13-oxooctadeca-9,11-dienoic acid and (9*E*,11*E*)-13-oxooctadeca-9,11-dienoic acid for peak *i* and *j*, respectively. Thus, signals of a CH_2−CH=CH−CH=CH−CO−CH_2 spin system were observed for the compound eluted as peak *i* with coupling constants corresponding to *Z*- and *E*-double bonds, respectively [$\delta_{H\text{-}8}$ 2.32 (qm, $J \approx 7.4$ Hz), $\delta_{C\text{-}8}$ 28.6; $\delta_{H\text{-}9}$ 5.93 (m), $\delta_{C\text{-}9}$ 142.8; $\delta_{H\text{-}12}$ 6.15 (d, J = 15.2 Hz), $\delta_{C\text{-}12}$ 130.6; $\delta_{H\text{-}10}$ 6.16 (br t, J = 10.7 Hz), $\delta_{C\text{-}10}$ 127.8; $\delta_{H\text{-}11}$ 7.48 (ddd, J = 15.6. 11.6 and 1.1 Hz), $\delta_{C\text{-}11}$ 137.3]. The compound eluted as peak *j* also exhibited signals of a CH_2−CH=CH−CH=CH−CO−CH_2 spin system, but with both double bonds in the *E*-configuration [$\delta_{H\text{-}8}$ 2.18 (m), $\delta_{C\text{-}8}$ 33.5; $\delta_{H\text{-}12}$ 6.07 (d, J = 15.7 Hz), $\delta_{C\text{-}12}$ 128.8; δ_H 6.22−6.24 (H-9 and H-10, m) correlated to δ_C 129.8 and 146.1; $\delta_{H\text{-}11}$ 7.15 (m), $\delta_{C\text{-}11}$ 143.4]. Both compounds exhibited triplets for H-14 (J = 7.5 Hz) at δ_H 2.58 and 2.64 (peak *i* and *j*, respectively) correlated to δ_C 40.7. The ^1H NMR spectra were in close agreement with literature data.[56] In positive-ion mode HRMS,

both compounds exhibited significant ions formed by a neutral loss of 116.0823 amu, corresponding to $C_6H_{12}O_2$ with formation of an ion with m/z 179.1429 ($[M - CH_3(CH_2)_4COOH + H]^+$, ΔM 0.8 ppm), resulting from cleavage of the C-6−C-7 bond (allylic cleavage of enolized dienone system). MS/MS of $[M - H]^-$ pseudomolecular ion of both compounds (peak *i*: m/z 293.2125, ΔM 0.8 ppm; peak *j*: m/z 293.2113, ΔM 3.1 ppm) displayed fragments, for peak *i* and *j*, respectively, at m/z 195.1389 and 195.1396 ($[M - CH_3(CH_2)_4CO - H]^-$, ΔM 0.8 and 2.6 ppm), 139.1133 and 139.1122 ($[M - CH_3(CH_2)_4COCHCH_2 - CO - H]^-$, ΔM 3.5 and 4.3 ppm), and 113.0974 and 113.0974 ($[M - CH_3(CH_2)_4COCHCHCHCH_2 - CO - H]^-$, ΔM 1.9 and 1.9 ppm). These fragments have been reported to be characteristic of 13-oxooctadeca-9,11-dienoic acids but are not observed for the corresponding 9-hydroxyoctadeca-10,12-dienoic acids.[56] This provides proof of the structure of compounds eluted as peaks *i* and *j*. The compound eluted as peak *k* (t_R = 55.8 min, m/z 281.2471, $[M + H]^+$ corresponding to $C_{18}H_{33}O_2^+$, ΔM 1.6 ppm) was linoleic acid [(9*Z*,12*Z*)-9,12-octadeca-9,12-dienoic acid], as confirmed by its ^{1}H NMR data.

After the identification of compounds accounting for peaks *a*−*k* by the hyphenated technique, an isolation procedure that targets compounds **1**−**4**, considered to be of interest as they represent rare carbon skeletons, could be designed. Since the compounds are glycosides of tertiary alcohols, they are acid-labile, and thus an acid-free mobile phase was used for chromatography even though the presence of acid improved separation and peak shapes of other extract constituents such as phenolics. The fractionation was monitored by positive-ion mode HRMS, collecting fractions that exhibited ions corresponding to m/z 369.2636 (compound **1**), 383.2428 (compounds **2** and **4**), and 385.2585 (compound **3**). Compound **5** was not targeted because of its presumed instability. This afforded pure samples of spiranes **1**−**4** in an optimized procedure.

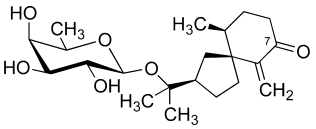
Interestingly, in addition to **1**−**4** the targeted procedure afforded a small amount of one additional spiro compound. This compound, subsequently identified as **6** (m/z 383.2430, $[M + H]^+$ corresponding to $C_{21}H_{35}O_6^+$, ΔM 0.5 ppm), is an isomer of **2** and **4**, and thus not only the fractions containing the latter but also those containing this spiroketone were classified as targets by HRMS and collected. The structure of the new spiroketone **6** follows from ^{1}H and ^{13}C NMR spectra (Table 2) supported by 2D NMR data. It is noteworthy that this compound was missed in the HPLC-PDA-HRMS-SPE-NMR analysis. Reexamination of the chromatograms of the crude extract showed that **6** was present as a minor component of the peak cluster eluted just after peak *e* (Figure 1). These small unresolved peaks were not selected for the initial HPLC-PDA-HRMS-SPE-NMR analysis, and thus compound **6** was not detected. This example highlights the importance of peak selection in HPLC-PDA-HRMS-SPE-NMR analysis applied as an exploratory tool prior to targeted isolation. Since it is not possible for a complex crude extract to achieve complete separation of all constituents, especially minor constituents, some compounds may be overlooked.

The example described in this work demonstrates the effectiveness of HPLC-PDA-HRMS-SPE-NMR as a preparatory stage for targeted isolation. The original, nontarget isolation took about 1.5 months of laboratory work and consumed large amounts (over 20 L) of organic solvents. This was mainly the result of pursuing numerous fractions, most of which turned out to contain unwanted compounds. This, however, became apparent only after a considerable fractionation effort. By contrast, targeted isolation focused on the spiranes was completed during a few days. Prior to that, approximately two weeks had to be spent on the hyphenated analysis. Most of this time was instrument time not requiring constant involvement of an operator. Chromatographic separations and peak trapping and elution were accomplished during two days. In summary, targeted isolation based on HPLC-PDA-HRMS-SPE-NMR is much more cost-effective as compared with nontarget isolation once the necessary hyphenation equipment is available. The spiranes isolated in this work obey Lipinski's rule of five (3 or 4 hydrogen bond donors, 5 or 6 hydrogen bond acceptors, MW 368−384, calculated log *P* 1.24−2.68).[59,60] They were included in our screening library of pure natural products.[61,62]

Table 2. NMR Data for Compound 6[a]

position	δ_C, type	δ_H (*J* in Hz)
1	34.5, CH_2	1.74, dd (13.7, 9.6)
		1.81, m
2	51.0, CH	2.08, m
3	26.4, CH_2	1.53, m
		1.60, m
4	39.3, CH_2	1.51, m
		1.62, m
5	53.7, C	
6	155.0, C	
7	204.1, C	
8	36.7, CH_2	2.32, ddd (17.2, 6.2, 3.4)
		2.47,ddd (17.2, 11.4, 7.4)
9	27.4, CH_2	1.67, ddt (13.8, 7.4, 3.7)
		2.11, m
10	38.6, CH	1.84, m
11	78.9, C	
12	24.8, CH_3	1.18, s
13	26.0, CH_3	1.22, s
14	117.9, CH_2	5.41, d (1.5)
		5.66, d (1.5)
15	15.8, CH_3	1.00, d (7.0)
1′	98.1, CH	4.33, d (7.7)
2′	72.3, CH	3.24, dd (9.5, 7.7)
3′	74.7, CH	3.40, dd (9.5, 3.6)
4′	72.3, CH	3.53, dd (3.6, 1.2)
5′	70.6, CH	3.56, dq (6.5, 1.2)
6′	16.9, CH_3	1.17, d (6.5)

[a]600 MHz spectra in acetonitrile-d_3; d, doublet; t, triplet; q, quartet; m, multiplet; ^{13}C chemical shifts from proton-detected 2D correlations.



6

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a Jasco DIP-370 polarimeter. NMR spectra were recorded at 300 K with a Bruker Avance III 600 MHz spectrometer equipped with a 5 mm TXI probe or a cryogenically cooled 1.7 mm TCI probe using methanol-d_4 or acetonitrile-d_3 (99.8 atom % of

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deuterium) as solvents. Mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer equipped with an ESI interface. Analytical HPLC analyses were performed on a Shimadzu LC-10A system consisting of a controller, a solvent supply unit, a diode array detector, an autosampler, and a column oven, using a Phenomenex Luna C18(2) column (4.6 mm × 150 mm, 3 μm) operated at 40 °C. Preparative HPLC analyses for the traditional approach were performed on an Agilent 1100 system equipped with two preparative solvent delivery units, a multiple wavelength detector, an autosampler, and an optional fraction collector, using a 21.2 mm × 250 mm Phenomenex Luna C18 (5 μm) operated at room temperature. Preparative HPLC analyses for targeted isolation were performed on a Shimadzu CMD-20A system consisting of a controller, two preparative solvent delivery units, a diode array detector, an autosampler, and a fraction collector, using a 21.2 mm × 250 mm Phenomenex Luna C18 (5 μm) operated at room temperature. The HPLC-PDA-HRMS-SPE-NMR system consisted of an Agilent 1100 chromatograph composed of a quaternary pump, a photodiode array detector, and an autosampler, a Bruker micrOTOF-Q II mass spectrometer equipped with an electrospray ionization source and operated via a 1:99 flow splitter, a Knauer Smartline 100 pump for postcolumn flow dilution, two Spark Holland Prospect 2 SPE units (one configured for trapping and the other for elution), a Gilson 215 liquid handler for automated filling of 1.7 mm NMR tubes from the Prospect 2 device configured for SPE cartridge elution, and the above-mentioned Bruker Avance III 600 MHz NMR spectrometer equipped with a cooled SampleJet sample managing device. The hyphenation experiments were performed using a Phenomenex Luna C18(2) column (4.6 mm × 150 mm, 5 μm) operated at 25 °C.

NMR Experiments. Spectra of samples from the hyphenation experiments, recorded in acetonitrile-d_3 in 1.7 mm NMR tubes at 300 K, were calibrated to solvent signals (δ 1.94 for residual ^{1}H, δ 1.32 for ^{13}C). 1D ^{1}H NMR spectra were recorded using 90° pulses with HOD signal saturation during relaxation delay (4.0 s). The spectra were obtained by summing 512 transients and acquiring 64k data points with a spectral width of 24 ppm. Phase-sensitive DQF-COSY spectra were recorded using a gradient-based pulse sequence with solvent suppression through excitation sculpting during relaxation delay (1.0 s), with a 12 ppm spectral width and 2k × 512 data points (processed with forward linear prediction to 1k data points). Phase-sensitive NOESY spectra were acquired with solvent suppression through excitation sculpting, spectral width 24 ppm, 2k × 256 data points (processed with forward linear prediction to 1k data points), 2.0 s relaxation delay, and a mixing time of 600 ms. Multiplicity-edited HSQC spectra were acquired with a spectral width of 24 ppm for ^{1}H and 185 ppm for ^{13}C and 2k × 256 data points (processed with forward linear prediction to 1k data points) and a 1.0 s relaxation delay. HMBC spectra (without a low-pass filter) were optimized for $^nJ_{C,H}$ = 8 Hz and acquired with a spectral width of 24 ppm for ^{1}H and 240 ppm for ^{13}C, 2k × 128 data points (processed with forward linear prediction to 1k), and 1.0 s relaxation delay. Samples from preparative isolation were analyzed in 5 mm NMR tubes at 300 K using 30° pulses and were calibrated to internal TMS. 1D ^{1}H NMR spectra were recorded summing 256 transients, acquiring 64k data points with a spectral width of 20 ppm and a relaxation delay of 1.0 s. Phase-sensitive DQF-COSY spectra were recorded using a gradient-based pulse sequence with purge pulses prior to relaxation delay (2.0 s), with spectral widths optimized for each sample and 2k × 256 data points (processed with forward linear prediction to 1k data points). Phase-sensitive NOESY spectra were acquired using a gradient-based pulse sequence with spectral widths optimized for each sample, 2k × 256 data points (processed with forward linear prediction to 1k data points), a relaxation delay of 2 s, and a mixing time of 300 ms. Multiplicity-edited HSQC spectra were acquired with a spectral width of 12 ppm for ^{1}H and 165 ppm for ^{13}C, 2k × 256 data points (processed with forward linear prediction to 1k data points), and a 2.0 s relaxation delay. HMBC spectra (with low-pass filter) were optimized for $^nJ_{C,H}$ = 8 Hz and acquired with spectral width optimized for each sample for ^{1}H and 220 ppm for ^{13}C, 4k × 256 data points (processed with forward linear prediction to 1k), and a 2.0 s relaxation delay.

Plant Material. *Carthamus oxyacantha* M. Bieb. (Asteraceae) was collected in Golestan National Park, northern Iran, N 37°28.459′, E 56°46.810′. The material was authenticated by Mr. Ali Ahi, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. A voucher specimen (DFHJJ51) was deposited in Herbarium C (Botanical Museum, University of Copenhagen, Denmark).

Extract Preparation. Ground material of *C. oxyacantha* (aerial parts, 174 g) was extracted with EtOH (2.1 L, then 2 × 1.5 L) by soaking at room temperature overnight. The extracts were filtered, pooled, concentrated in vacuo, and dried on a freeze-dryer to give 10.5 g of crude extract. The crude extract was divided into two portions for the traditional fractionation procedure and for the hyphenated experiments (9.5 and 1.0 g, respectively).

Nontarget Preparative Fractionation. The crude extract (9.5 g) was fractionated by means of VLC (11 × 10 cm i.d., column, 365 g of silica gel 60, 0.015−0.040 mm, from Merck). After defatting with petroleum ether (bp 40−60 °C, 800 mL) and petroleum ether−EtOAc (70:30, 800 mL), the column was eluted with EtOAc, EtOAc−MeOH (80:20), EtOAc−MeOH (50:50), and MeOH (800 mL of each), yielding four fractions (fractions A−D; 1.24, 2.0, 2.15, and 0.85 g, respectively). The four fractions were further investigated by preparative HPLC (see General Experimental Procedures) with linear solvent gradients composed of MeCN−H_2O (5:95) + 0.1% CF_3COOH (eluent A) and MeCN−H_2O (95:5) + 0.1% CF_3COOH (eluent B) delivered at 20 mL/min; after evaporation in vacuo the HPLC fractions were investigated by NMR spectroscopy. A portion of fraction A (377 mg) was resolved by HPLC using a gradient profile from 5% to 50% of B over 30 min to give 10 fractions (A1−A10) containing fatty acids (^{1}H NMR). Fraction B (HPLC of 480 mg portion using a gradient from 10% to of 80% of eluent B over 30 min) gave 12 fractions, B1−B12. Fraction B1 contained 2 mg of vanillic acid. Fraction B5 yielded 8 mg of tracheloside. Fraction B7 gave 8.5 mg of compound **2**. Fraction B8 gave 9 mg of compound **3**.[31] Fraction B9 gave 7 mg of compound **4**, and fraction B11 contained 75 mg of compound **1**.[25] The remaining fractions contained flavonol and chalcone glycosides and caffeic acid derivatives (^{1}H NMR). Fraction C (HPLC of 1400 mg portion using a gradient profile from 5% to 50% of eluent B in 30 min) yielded eight fractions, C1−C8. Fraction C6 contained 4 mg of (2*E*,4*R*)-4-hydroxynon-2-enoic acid,[30,31] whereas the remaining fractions contained flavonoids. Fraction D (HPLC of 280 mg portion using a gradient profile from 0% to 60% of B in 30 min) was resolved to fractions D1−D6, of which D1 was 4-(β-D-glucopyranosyloxy)-3-methoxybenzoic acid[32] (1 mg), whereas the remaining fractions did not yield any well-defined products.

HPLC-PDA-HRMS-SPE-NMR Analysis. The crude extract of *C. oxyacantha* (1 g) was dissolved in MeOH−H_2O (9:1, 100 mL) and extracted three times with petroleum ether (50 mL each time), the defatted solution was evaporated, and the residue was redissolved in MeOH (20 mg/mL). The mobile phase used was composed of MeCN−H_2O (5:95) + 0.1% HCOOH (eluent A) and MeCN−H_2O (95:5) + 0.1% HCOOH (eluent B), using a linear gradient profile from 20% to 100% of B in 50 min at 0.5 mL/min. The injection volume was 25 μL. The chromatography was monitored by MS (base-peak chromatogram) and PDA detector (210, 254, and 380 nm), using thresholds of the first three signals to trigger SPE trapping. The HPLC eluate was diluted with H_2O (1.0 mL/min) prior to trapping on Spark Holland 2 × 10 mm GP-resin SPE cartridges, conditioned with 500 μL of MeCN at 6 mL/min, and equilibrated with 500 μL of H_2O at 1 mL/min. The cartridges were dried with pressurized N_2 for 30 min, and the analytes were eluted with 30 μL of CD_3CN into 1.7 mm NMR tubes. The total transfer volume was 187 at 100 μL/min. Initial trappings were conducted with 30 peaks to acquire 1D ^{1}H NMR spectra. Subsequently, multiple cumulative trapping (eight trappings) was performed with 11 peaks to acquire sets of 2D data (DQF-COSY, NOESY, HSQC, HMBC).

Targeted Isolation. Ground material of *C. oxyacantha* (aerial parts, 20 g) was extracted with EtOH (400 mL, then 2 × 300 mL) by

soaking at room temperature overnight. The extracts were filtered, pooled, concentrated in vacuo, and dried on a freeze-dryer to give 2.0 g of crude extract. The material was dissolved in MeOH−H_2O (9:1, 100 mL) and extracted three times with petroleum ether (100 mL each time). The defatted extract was fractionated by means of VLC (4.5 × 4 cm i.d., 25 g of silica gel 60, 0.015−0.040 mm). The column was eluted with EtOAc (100 mL) followed by a stepwise gradient of MeOH in EtOAc, rising from 1% to 6% by volume in 1% steps, using 2 × 25 mL in each step. Each fraction was analyzed by HRMS for the presence of the targeted compounds. Fractions were pooled to yield seven fractions containing mixtures of compounds **1**−**4** in various amounts. The fractions were further separated by preparative HPLC with a linear solvent gradient composed of MeCN−H_2O (5:95) (eluent A) and MeCN−H_2O (95:5) (eluent B) from 0% to 80% of B over 25 min, delivered at 20 mL/min. This yielded, after pooling as appropriate, compound **1** (52.4 mg), **2** (7.6 mg), **3** (1.3 mg), **4** (3.3 mg), and **6** (1.6 mg).

(2*R,5*S**,10*S**)-6,10-Dimethyl-2-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]spiro[4.5]dec-6-ene (Hinesol β-D-fucopyranoside) (1):** $[\alpha]^{25}_D$ −34, *c* 0.2 in MeOH, lit.[25] $[\alpha]^{24}_D$ −35.5, *c* 3.5 in $CHCl_3$; ^1H and ^{13}C NMR data recorded in hyphenation mode, see Table 1; HR-ESIMS(+) *m*/*z* 369.2642 (calcd for $C_{21}H_{37}O_5^+$, 369.2636).

(2*R,5*S**,10*S**)-6,10-Dimethyl-2-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]spiro[4.5]dec-6-en-8-one (2):** $[\alpha]^{25}_D$ −55, *c* 0.3 in MeOH; ^1H and ^{13}C NMR data recorded in hyphenation mode, see Table 1; HR-ESIMS(+) *m*/*z* 383.2426 (calcd for $C_{21}H_{35}O_6^+$, 383.2428).

(2*R,5*S**,7*S**,10*S**)-10-Methyl-2-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]- 6-methylenespiro[4.5]decan-7-ol (3):** $[\alpha]^{25}_D$ −47, *c* 0.04 in MeOH; ^1H and ^{13}C NMR data recorded in hyphenation mode, see Table 1; HR-ESIMS(+) *m*/*z* 385.2579 (calcd for $C_{21}H_{37}O_6^+$, 385.2585).

(4*S,5*S**,7*R**)-1,4-Dimethyl-7-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]spiro[4.4]non-1-ene-2-carbaldehyde (4):** $[\alpha]^{25}_D$ −33, *c* 0.08 in MeOH; ^1H and ^{13}C NMR data recorded in hyphenation mode, see Table 1; HR-ESIMS(+) *m*/*z* 383.2424 (calcd for $C_{21}H_{35}O_6^+$, 383.2428).

(2*R,5*S**,7*R**,10*S**)-7-Hydroperoxy-2-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]-10-methyl-6-methylenespiro[4.5]-decane (5):** ^1H and ^{13}C NMR data recorded in hyphenation mode, see Table 1; HRESIMS(+) *m*/*z* 401.2536 (calcd for $C_{21}H_{37}O_7^+$, 401.2534).

(2*R,5*S**,10*S**)-10-Methyl-6-methylene-2-[(1-methyl-1-β-D-fucopyranosyloxy)ethyl]spiro[4.5]decan-8-one (6):** $[\alpha]^{25}_D$ −16, *c* 0.06 in MeOH; ^1H and ^{13}C NMR data, see Table 2; HR-ESIMS(+) *m*/*z* 383.2430 (calcd for $C_{21}H_{35}O_6^+$, 383.2428).

4-(β-D-Glucopyranosyloxy)-3-methoxybenzoic acid: $[\alpha]^{25}_D$ −50, *c* 0.05 in MeOH; ^1H and ^{13}C NMR as previously reported,[32] the position of ring substituents confirmed by NOESY and HMBC; HR-ESIMS(+) *m*/*z* 331.1023 (calcd for $C_{14}H_{19}O_9^+$, 331.1024).

4-Hydroxy-3-methoxybenzoic Acid (vanillic acid): ^1H and ^{13}C NMR as previously reported,[63,64] the position of methoxy group confirmed by HMBC; HR-ESIMS(+) *m*/*z* 169.0497 (calcd for $C_8H_9O_4^+$, 169.0495).

(2*Z*,4*R*)-Hydroxynon-2-enoic acid: $[\alpha]^{25}_D$ −21, *c* 0.2 in MeOH, lit.[31] $[\alpha]^{23}_D$ −28 for material with 94% ee, *c* 1.0 in $CHCl_3$; ^1H and ^{13}C NMR data corresponding to those reported;[30,31] HR-ESIMS(+) *m*/*z* 173.1167 (calcd for $C_9H_{17}O_3^+$, 173.1172).

Nonanedioic Acid (azelaic acid): ^1H and ^{13}C NMR data recorded in hyphenation mode corresponding to those reported;[65] HR-ESIMS(+) *m*/*z* 189.1119 (calcd for $C_9H_{17}O_4^+$, 189.1121).

Tracheloside: $[\alpha]^{29}_D$ −49, *c* 0.4 in MeOH, lit.[28] $[\alpha]^{20}_D$ −60, *c* 0.5 in EtOH; ^1H and ^{13}C NMR data, see supplementary Table S1; HR-ESIMS(+) *m*/*z* 551.2121 (calcd for $C_{27}H_{35}O_{12}^+$, 551.2123).

(10*E*,12*Z*)-9-Hydroxyoctadeca-10,12-dienoic acid (α-dimorphecolic acid): ^1H and ^{13}C NMR data recorded in hyphenation mode corresponding to those reported,[51] see also main text; HR-ESIMS(−) *m*/*z* 295.2276 (calcd for $C_{18}H_{31}O_3^-$, 295.2279).

(9*Z*,11*E*)-13-Oxooctadeca-9,11-dienoic acid: ^1H and ^{13}C NMR data recorded in hyphenation mode corresponding to those reported,[56] see also main text; HR-ESIMS(−) *m*/*z* 293.2125 (calcd for $C_{18}H_{29}O_3^-$, 293.2122).

(9*E*,11*E*)-13-Oxooctadeca-9,11-dienoic acid: ^1H and ^{13}C NMR data recorded in hyphenation mode corresponding to those reported,[56] see also main text; HR-ESIMS(−) *m*/*z* 293.2113 (calcd for $C_{18}H_{29}O_3^-$, 293.2122).

(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid (linoleic acid): ^1H and ^{13}C NMR data recorded in hyphenation mode corresponding to those reported;[66] HR-ESIMS(+) *m*/*z* 281.2471 (calcd for $C_{18}H_{33}O_2^+$, 281.2475).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra of compounds **2**, **4**, **5**, and **6** and NMR data for tracheloside. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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