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Homologous very-long-chain 1,3-alkanediols and 3-hydroxyaldehydes in leaf cuticular waxes of *Ricinus communis* L.

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

Surface extracts from primary leaves of Castor bean were found to contain 1.8 μ g cm^{-2} of cuticular waxes. The mixture comprised alkanes (C_{26}–C_{29}), primary alcohols (C_{22}–C_{38}), aldehydes (C_{26} and C_{28}), fatty acids (C_{20}–C_{34}) and triterpenoids (lupeol, β- and α-amyrin). Besides, a series of *n*-alkane-1,3-diols was detected, with chain lengths ranging from C_{22} to C_{28}, a strong predominance of even-numbered homologs, and a maximum for hexacosane-1,3-diol. Seven other compounds were assigned to a novel class of wax constituents and identified as homologous unbranched 3-hydroxyaldehydes ranging from C_{22} to C_{28}. As the chain length distribution of this series closely paralleled the homolog pattern of 1,3-diols, it seems likely that both compound classes are biosynthetically related.

Keywords: Cuticular waxes; Castor bean; *Ricinus communis*; Euphorbiaceae; Diols; Hydroxyaldehydes

1. Introduction

All primary aboveground plant organs are covered with cuticles consisting of a matrix of cutin that is impregnated with 'intracuticular waxes' and covered with 'epicuticular waxes'. Diverse biological functions have been attributed to these waxes: preventing transpirational water loss over the large plant surface (Schönherr, 1976), guarding leaf surfaces from accumulation of air-borne particles and spores (Barthlott and Neinhuis, 1997), directing the behavior of herbivorous insects that probe the surface for infochemicals (Espelie et al., 1991; Eigenbrode and Espelie, 1995), keeping leaf surfaces dry (Holloway, 1970) and, thus, preventing the germination of pathogen spores (Kolattukudy, 1980; Deising et al., 1992).

The specific physiological and ecological functions of plant cuticles can only be understood on the basis of their characteristic wax composition and biosynthetic origin. Typical wax mixtures comprise unbranched, saturated very-long chain aliphatics with only one oxygen-containing functional group. Previous reports on plant wax constituents carrying two or more functional groups are scarce, except for mid-chain β-diketones (Baker, 1982). In those cases reported, the relative position and nature of the functional groups allowed to infer (novel) biosynthetic pathways of wax compounds (Franich et al., 1979; Jetter et al., 1996; Jetter and Riederer, 1999; Jetter, 2000; Schulz et al., 2000; von Wettstein-Knowles, 1976). These hypotheses may now be further substantiated by detecting potential pathway intermediates. Hence, the objective of the present work was to identify and quantify series of biosynthetically related bifunctional compound classes. To this end, leaf cuticular waxes of *Ricinus communis* L. were analyzed using diverse chemical transformations and product structure assignment by GC–MS.

2. Results and discussion

In the surface extracts from mature primary leaves of *R. communis* various typical plant wax constituents were identified, including very-long-chain fatty acids, primary alcohols, aldehydes, and alkanes. According to their GC retention behavior and MS characteristics, several other major compounds could be assigned to two homologous series A and B. For structure elucidation, series A was purified from the extraction mixture by TLC (R_f

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0.03, migrating together with fatty acids) and transformed into TMSi derivatives. A synthetic standard of tetracosane-1,3-diol proved to be identical with one constituent of fraction A, both in GC co-injection experiments and according to the TMSi ether MS. Corresponding fragmentation patterns finally served to unambiguously identify all the compounds in series A as homologous unbranched alkane-1,3-diols with chain lengths between C_{22} and C_{28}. These compounds had previously been described as cuticular wax constituents of *Cucumis sativus* (Fauth et al., 1998) and of *Papaver orientale* (Jetter et al., 1996), and as biological markers in miocene sediments (Huang et al., 1994).

Constituents of compound class B were located in a TLC band (R_f 0.13) migrating between primary alcohols (R_f 0.20) and alkanediols, and were therefore expected to contain two (or more) functional groups of intermediate polarity. In order to determine the number and position of these groups, the fraction was reduced with $LiAlH_4$ and transformed into TMSi derivatives. The GC–MS characteristics of the resulting compounds (Fig. 1A) were identical to those of TMSi ethers of the alkane-1,3-diols in fraction A and the standard of tetracosane-1,3-diol. The bifunctional structure of the unknown compounds was therefore confirmed, both oxygen-containing groups residing on positions 1 and 3 of an unbranched aliphatic chain. All alkane-1,3-diols ranging from C_{22} to C_{28} were identified, with a strong predominance of even-numbered homologs and a maximum for C_{26}.

In a consecutive experiment, the functional groups of compound class B were further characterized by TMSi derivatization without prior reduction. The resulting MS (Fig. 1B) showed the alcohol fragments m/z 75 and 103 but lacked a diol signal m/z 147, implying the presence of a single hydroxyl function. The prominent fragment m/z 145, detected for all homologs, could then be interpreted as an α-ion containing the hydroxyl function. Taking the 1,3-position of functional groups into account (see above), the structure $[(TMSiO)\text{-}C_3H_5O]^+$ of the α-fragment and, hence, the presence of a carbonyl function was inferred. Another fragment, varying for all homologs in fraction B, was interpreted as an α-ion $[C_nH_{2n+1}OTMSi]^+$ containing a long alkyl terminus. Both α-ions together showed that the hydroxyl function was located in 3-position. The

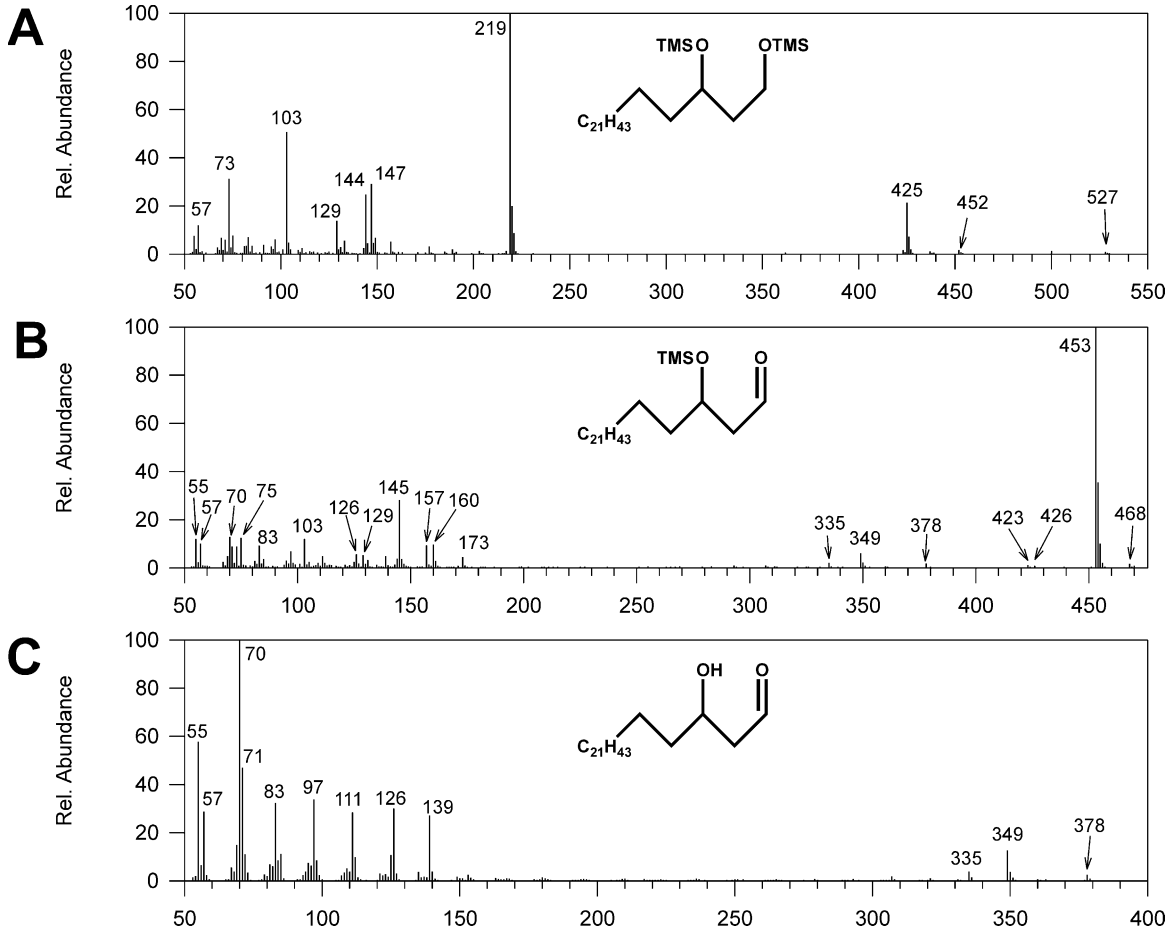

Fig. 1. Mass spectra of one representative 3-hydroxyaldehyde and its derivatives. For 3-hydroxyhexacosanal, (A) the bis TMSi ether of the reduction product (alkane-1,3-diol), (B) the TMSi ether, and (C) the underivatized compound are shown.

overall molecular geometry, i.e. the presence of one hydroxyl and one carbonyl function, was confirmed by corresponding signals for $[M]^+$, $[M-CH_3]^+$ and $[M-HOTMSi]^+$. Based on all the described evidence, the compounds in fraction B were tentatively identified as a homologous series of unbranched 3-hydroxyaldeydes. All homologs with chain lengths between C_{22} and C_{28} were identified and, in accordance with the reduction experiment, even-numbered representatives prevailed.

The underivatized hydroxyaldehydes were characterized by ions $[M-H_2O]^+$ representing α,β-unsaturated aldehydes (Fig. 1C). The latter products were in all spectra accompanied by fragments generated by loss of CHO, characteristic for unsaturated aldehydes (Li et al., 1999), and by series of ions *m*/*z* 55, 69, 83, etc. due to alkenyl decay. Two fragments in this series, *m*/*z* 69 and 125, showed relatively low abundance and instead even-mass ions *m*/*z* 70 and 126 prevailed. This can be attributed to the formation of stable cyclic fragments, probably with dihydrofuran geometry (Chang et al., 1990). Hence, the MS characteristics of the underivatized compounds in fraction B confirmed their 3-hydroxyaldehyde structure.

Finally, synthetic 3-hydroxytetracosanal was prepared to verify the structure assignment. Both the underivatized standard and its TMSi derivative showed GC characteristics and MS fragmentation patterns identical to one compound in fraction B. This homolog was hence unambiguously identified as the unbranched C_{24} 3-hydroxyaldehyde, ruling out all isomeric alternatives. From MS comparisons of synthetic 3-hydroxytetradecanal, 3-hydroxyhexadecanal and 3-hydroxyoctadecanal (and their TMSi derivatives) chain-length specific fragments could be deduced. Thus, the assignment of all hydroxyaldehydes in fraction A was confirmed, all seven homologs with chain lengths between C_{22} and C_{28} representing novel compounds.

The absolute coverages of all constituents in the total wax extract were quantified. Mature primary leaves of *R. communis* had total surface areas averaging at 122 cm^2 and yielded 1.8 μg cm^{-2} of wax. Besides, app. 0.9 μ g cm^{-2} of the alkaloid ricinin was extracted. Within the triterpenoid fraction (9.5% of the wax mixture), lupeol (5.1%), β-amyrin (2.6%) and α-amyrin (1.5%) were identified. The wax mixture was dominated by primary alcohols (48.1%) and alkanediols (10.6%). The novel hydroxyaldehydes amounted to 7.2% of the wax, while alkanes, aldehydes and fatty acids accounted for 2.3%, 0.9% and 3.9%, respectively (Table 1). A number of wax constituents, present in very small individual percentages, could not be identified (17.6%). Only in the alkanes odd-numbered homologs were prevailing, with a maximum for C_{27}. In the alcohol and aldehyde fractions C_{28} compounds predominated, while the fatty acids had a relatively broad homolog maximum from C_{22} to C_{30}.

In contrast to all other compound classes, the alkanediols and the hydroxyaldehydes had chain length distributions with a maximum for C_{26}. Based on the similarity of homolog patterns and the identical position of functional groups, it seems very likely that these two compound classes are biosynthetically related. We hypothesize that the 3-hydroxyaldehydes are formed by reduction of β-hydroxy acyl derivatives, possibly fatty acid elongation cycle intermediates. The alkane-1,3-diols could then be generated by reduction of the 3-hydroxyaldehydes, analogous to the well-characterized transformation of wax aldehydes into wax alcohols (von Wettstein-Knowles, 1995). Consequently, both 3-hydroxyaldehydes and 1,3-diols could be classified as very-long-chain polyketides similar to other plant wax constituents, e.g. β-diketones (von Wettstein-Knowles, 1976) and *sec*./*sec*. β-diols (Jetter, 2000). In conclusion, the novel hydroxyaldehydes would represent intermediates in a polyketide elongation–modification pathway that runs parallel to normal wax biosynthesis.

3. Experimental

3.1. Plant material and sample preparation

Plants of *R. communis* were continuously grown from seeds in greenhouses of the University of Würzburg. Four mature primary leaves were cut 19 days after germination and immediately immersed twice for 30 s in $CHCl_3$ at room temp. Both solns. were combined and a defined amount of *n*-tetracosane was added as internal standard. The solns were dried, filtered and concentrated under reduced pressure. The extracted leaf area was determined gravimetrically using photocopies of the leaves.

3.2. Wax analysis

For identification of unknown compounds, the wax mixture was separated by TLC (sandwich technique, silica gel, mobile phase $CHCl_3$–EtOH (99:1), staining with primuline and UV-light). Bands were immediately removed from the plates, eluted with $CHCl_3$, and filtered. Finally, the solvent was removed in a stream of N_2 and samples were stored in the dark at 4 °C. Two bands, designated as fractions A (R_f 0.03) and B (R_f 0.13), contained unknown compounds and were subjected to detailed qualitative analyses. To this end, either the underivatized constituents or their derivatization products were analyzed using GC (30 m OV-1 WCOT i.d. 320 μm, on-column-injection at 50 °C, oven 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, 30 min at 320 °C and He carrier gas inlet pressures 1 min at 5 kPa, 4 kPa min^{-1} to 18 kPa, 0,6 kPa min^{-1} to 40 kPa, 37 min at 40 kPa) with

Table 1
Composition of cuticular wax on mature primary leaves of *Ricinus communis*[a]

Chain length	Alkanes	Alcohols	Aldehydes	Fatty acids	Alkanediols	Hydroxyaldehydes
20				3.4±1.00		
22		2.3±0.22		24.3±7.11	10.3±0.60	2.6±0.29
23		Tr			2.1±1.60	0.2±0.04
24		3.0±0.86		12.2±1.77	22.8±0.93	13.8±0.53
25		0.9±0.20			4.1±0.15	2.7±0.13
26	8.5±6.76	20.8±0.56	35.1±8.36	17.3±4.75	50.6±1.11	63.2±0.66
27	54.2±1.53	2.7±0.14			1.3±0.39	2.4±0.60
28	13.0±5.03	44.6±1.53	64.9±8.36	11.6±2.76	8.8±0.58	15.1±0.91
29	24.2±1.32	n.d.				
30		15.4±0.07		19.2±2.70		
31		0.7±0.06				
32		4.9±0.16		6.1±1.39		
33		0.3±0.04				
34		2.2±0.12		5.9±1.50		
36		1.3±0.02				
38		1.0±0.07				
Total	2.3±0.09	48.1±2.14	0.9±2.14	3.9±1.01	10.6±1.03	7.2±0.29

[a] The relative quantities (%) of individual homologs within compound classes and the total percentages of these fractions in the wax mixture are given as mean values of three independent parallels (±S.D.).

MS detection (70eV, *m*/*z* 50–650). Constituents of fractions A and B were derivatized in two alternative reactions. (1) Compounds containing free hydroxyl groups were transformed into TMSi ethers by reaction with bis-*N*,*N*-trimethylsilyltrifluoroacetamide in pyridine 30 min at 70 °C. (2) Carbonyl groups were reduced by addition of $LiAlH_4$ in refluxing THF over 24 h. The mixt. of LiAl-alcoholate complexes was hydrolyzed with 10% H_2SO_4, the alcohols obtained by extraction of the soln with Et_2O and transformed into the corresponding TMSi ethers.

For quantitative analysis of the whole wax mixture hydroxyl groups were transformed to TMSi derivatives and constituents were identified using GC–MS (as above). Area coverages of individual compounds were then determined by GC–FID (as above, but H_2 carrier gas inlet pressures 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, 50 min at 150 kPa) and values for compound classes were calculated by adding those of all identified homologs. Leaf surface coverages of individual constituents were calculated as mean values of three independent analyses.

3.3. Synthesis of reference compounds

Docosanoic acid (Fluka, Deisenhofen, Germany) was transformed into 3-oxotetracosanoic acid ethyl ester employing common procedures for malonate synthesis (Kobajashi et al., 1984). The 3-oxoacid ester was reduced to tetracosane-1,3-diol using $LiAlH_4$ as described above. Alternatively, oxotetracosanoic acid ethyl ester was reduced to 3-hydroxytetracosanal via the imidazolide (Staab and Bräunling, 1962). To a solution of 15 μmol oxoacid ester in 400 μl of dry THF at room temp., first 20 μmol of *N*,*N*′-carbonyldiimidazol and after 60 min 10 μmol of $LiAlH_4$ were added. After 60 min the reaction was stopped with H_2O, the product obtained by extraction of the soln. with Et_2O, purified by TLC, and transformed into the corresponding TMSi ether. The same procedure served to transform commercial 3-hydroxytetradecanoic acid, 3-hydroxyhexadecanoic acid and 3-hydroxyoctadecanoic acid (CPS, Düren, Germany) into the corresponding hydroxyaldehydes.

3.4. Mass spectral data of selected compounds

Underivatized hydroxyaldehydes *m*/*z* (rel int.): 3-Hydroxytetracosanal $[M-H_2O]^+$ 350 (1), $[M-H_2O-CHO]^+$ 321 (15), $[M-61]^+$ 307 (4), $[C_9H_{15}O]^+$ 139 (31), $[C_8H_{13}O]^+$ 126 (24), $[C_7H_{11}O]^+$ 111 (21), $[C_6H_9O]^+$ 97 (29), $[C_5H_7O]^+$ 83 (28), $[C_4H_6O]^+$ 70 (100), $[C_3H_3O]^+$ 55 (59). 3-Hydroxypentacosanal $[M-H_2O]^+$ 364 (1), $[M-H_2O-CHO]^+$ 335 (15), $[M-61]^+$ 321 (7), $[C_9H_{15}O]^+$ 139 (21), $[C_8H_{13}O]^+$ 126 (20), $[C_7H_{11}O]^+$ 111 (22), $[C_6H_9O]^+$ 97 (24), $[C_5H_7O]^+$ 83 (35), $[C_4H_6O]^+$ 70 (100), $[C_3H_3O]^+$ 55 (50). 3-Hydroxyhexacosanal $[M-H_2O]^+$ 378 (2), $[M-H_2O-CHO]^+$ 349 (12), $[M-61]^+$ 335 (4), $[C_9H_{15}O]^+$ 139 (26), $[C_8H_{13}O]^+$ 126 (29), $[C_7H_{11}O]^+$ 111 (29), $[C_6H_9O]^+$ 97 (35), $[C_5H_7O]^+$ 83 (34), $[C_4H_6O]^+$ 70 (100), $[C_3H_3O]^+$ 55 (60). 3-Hydroxyheptacosanal $[M-H_2O]^+$ 392 (1), $[M-H_2O-CHO]^+$ 363 (8), $[M-61]^+$ 349 (2), $[C_9H_{15}O]^+$ 139 (28), $[C_8H_{13}O]^+$ 126 (29), $[C_7H_{11}O]^+$ 111 (25), $[C_6H_9O]^+$ 97 (37), $[C_5H_7O]^+$ 83 (36), $[C_4H_6O]^+$ 70 (100), $[C_3H_3O]^+$

55 (63). 3-Hydroxyoctacosanal $[M-H_2O]^+$ 406 (1), $[M-H_2O-CHO]^+$ 377 (11), $[M-61]^+$ 363 (3), $[C_9H_{15}O]^+$ 139 (27), $[C_8H_{13}O]^+$ 126 (34), $[C_7H_{11}O]^+$ 111 (39), $[C_6H_9O]^+$ 97 (41), $[C_5H_7O]^+$ 83 (42), $[C_4H_6O]^+$ 70 (100), $[C_3H_3O]^+$ 55 (62).

TMSi ethers of hydroxyaldehydes m/z (rel int.): 3-Hydroxydocosanal TMSi ether $[M]^+$ 412 (1), $[M-CH_3]^+$ 397 (100), 370 (1), 367 (1), $[M-HOTMSi]^+$ 322 (1), $[M-HOTMSi-CHO]^+$ 293 (7), 279 (1), 173 (4), 160 (8), 157 (8), $[C_3H_4O_2(TMSi)]^+$ 145 (26), 139 (4), 129 (6), 126 (5), 111 (6), 103 (14), 97 (13), 83 (10), $[TMSi]^+$ 75 (13), 73 (10), 57 (15), 55 (12). 3-Hydroxytetracosanal TMSi ether $[M]^+$ 440 (1), $[M-CH_3]^+$ 425 (100), 398 (1), 395 (1), $[M-HOTMSi]^+$ 350 (1), $[M-HOTMSi-CHO]^+$ 321 (5), 307 (2), 173 (4), 160 (8), 157 (9), $[C_3H_4O_2(TMSi)]^+$ 145 (26), 139 (5), 129 (5), 126 (5), 111 (4), 103 (10), 97 (6), 83 (9), $[TMSi]^+$ 75 (13), 73 (8), 70 (13), 57 (10), 55 (11). 3-Hydroxypentacosanal TMSi ether $[M]^+$ 454 (1), $[M-CH_3]^+$ 439 (100), 412 (1), 409 (1), $[M-HOTMSi]^+$ 364 (1), $[M-HOTMSi-CHO]^+$ 335 (4), 321 (2), 173 (4), 160 (8), 157 (6), $[C_3H_4O_2(TMSi)]^+$ 145 (20), 139 (5), 129 (13), 126 (6), 111 (7), 103 (8), 97 (9), 83 (9), $[TMSi]^+$ 75 (11), 73 (11), 70 (16), 57 (12), 55 (12). 3-Hydroxyhexacosanal TMSi ether $[M]^+$ 468 (2), $[M-CH_3]^+$ 453 (100), 426 (1), 423 (1), $[M-HOTMSi]^+$ 378 (2), $[M-HOTMSi-CHO]^+$ 349 (6), 335 (2), 173 (4), 160 (10), 157 (9), $[C_3H_4O_2(TMSi)]^+$ 145 (28), 139 (5), 129 (5), 126 (5), 111 (5), 103 (12), 97 (7), 83 (9), $[TMSi]^+$ 75 (12), 73 (9), 70 (13), 57 (10), 55 (12). 3-Hydroxyheptacosanal TMSi ether $[M]^+$ 482 (1), $[M-CH_3]^+$ 467 (100), 440 (1), 437 (1), $[M-HOTMSi]^+$ 392 (3), $[M-HOTMSi-CHO]^+$ 363 (3), 173 (6), 160 (15), 157 (13), $[C_3H_4O_2(TMSi)]^+$ 145 (37), 139 (8), 129 (4), 126 (9), 111 (7), 103 (14), 97 (6), 83 (12), $[TMSi]^+$ 75 (18), 73 (8), 70 (17), 57 (27), 55 (11). 3-Hydroxyoctacosanal TMSi ether $[M]^+$ 496 (2), $[M-CH_3]^+$ 481 (100), 454 (1), 451 (1), $[M-HOTMSi]^+$ 406 (3), $[M-HOTMSi-CHO]^+$ 377 (10), 173 (6), 160 (12), 157 (11), $[C_3H_4O_2(TMSi)]^+$ 145 (33), 139 (4), 129 (7), 126 (10), 111 (8), 103 (14), 97 (11), 83 (14), $[TMSi]^+$ 75 (19), 73 (10), 70 (21), 57 (17), 55 (13).

Hydroxyaldehyde reduction products *m/z* (rel int.): Docosane-1,3-diol TMSi ether and tetracosane-1,3-diol bis TMSi ether as reported (Jetter et al., 1996). Tricosane-1,3-diol bis TMSi ether $[M-CH_3]^+$ 485 (2), $[M-C_2H_4OTMSi]^+$ 383 (18), $[C_3H_5(TMSiO)_2]^+$ 219 (100), 147 (25), 144 (21), 129 (55), $[CH_2OTMSi]^+$ 103 (47), $[TMSi]^+$ 73 (52), 57 (7). Pentacosane-1,3-diol bis TMSi ether $[M-CH_3]^+$ 513 (1), $[M-C_2H_4OTMSi]^+$ 411 (24), $[C_3H_5(TMSiO)_2]^+$ 219 (100), 147 (26), 144 (20), 129 (11), $[CH_2OTMSi]^+$ 103 (43), $[TMSi]^+$ 73 (28), 57 (9). Hexacosane-1,3-diol bis TMSi ether $[M-CH_3]^+$ 527 (0.8), $[M-C_2H_4OTMSi]^+$ 425 (25), $[C_3H_5(TMSiO)_2]^+$ 219 (100), 147 (28), 144 (26), 129 (12), $[CH_2OTMSi]^+$ 103 (48), $[TMSi]^+$ 73 (32), 57 (13). Heptacosane-1,3-diol bis TMSi ether $[M-CH_3]^+$ 541 (0.7), $[M-C_2H_4OTMSi]^+$ 439 (20), $[C_3H_5(TMSiO)_2]^+$ 219 (100), 147 (24), 144 (15), 129 (10), $[CH_2OTMSi]^+$ 103 (42), $[TMSi]^+$ 73 (20), 57 (10). Octacosane-1,3-diol bis TMSi ether $[M-CH_3]^+$ 555 (0.4), $[M-C_2H_4OTMSi]^+$ 453 (18), $[C_3H_5(TMSiO)_2]^+$ 219 (100), 147 (26), 144 (23), 129 (12), $[CH_2OTMSi]^+$ 103 (41), $[TMSi]^+$ 73 (27), 57 (11).

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