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# Study of Croatian non-aromatic naphthalane constituents with skeletons analogous to bioactive compounds☆

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**Abstract**

Non-aromatic Croatian naphthalane (NAN), shown to be efficace in the treatment of psoriasis vulgaris, was studied in order to improve our understanding of its constituents, which may be potentially responsible for its bioactivity. The components steranes and hopanes were analysed. Since NAN is a complex mixture of hydrocarbons, high-resolution GC and GC–MS were applied as the methods of choice in the study. The GC chromatogram of NAN showed a remarkable cluster in which sterane peaks prevail and composed 33±1% of the sample. Identified steranes (by GC–MS) represented almost half of the cluster (48%). They were in the range from norcholestanes up to propyl cholestanes. The amount of α-steranes (8.9%) was higher than of β-steranes (6.4%) and regular steranes (15.3%) dominated in the ratio 17:1 over the rearranged ones (0.9%). Steranes conserved the skeleton of bio-precursors and remained analogues of bioactive compounds, such as of vitamins D and some hormones and corticosteroids. Pentacyclic hydrocarbons hopanes, as derivatives of bacteriohopantetraol which is the physiological equivalent of cholesterol, made 4.7±0.2% of NAN. 

*Keywords:* Naphthalane preparations; Petroleum; Steranes; Hopanes

## 1. Introduction

Naphthalanes are medical preparations derived from a specific type of petroleum. They have been used for accelerating the regeneration of damaged skin and mucous membrane, for healing some diseases of peripheral nerve system, for some locomotion diseases, and for some blood tube diseases.

Since naphthalane preparations are used in human application, their composition needs to be studied as much in detail as possible. In an ideal case, each single component should be identified and the bioactivity of each single component should be determined, and in addition all components' mutual interactions should be learnt. The naphthalane composition complexity, mirrored in hundreds of peaks [resolved by high-resolution GC (HRGC)], represents a serious obstacle to reaching this goal. Due to

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this complexity, only the focused study of selected constituents is possible.

A non-aromatic Croatian naphthalane preparation (NAN) which met the requirements of Ph. Eur. [1] has been recently studied [2,3]. It was shown [2] to be composed exclusively of genuine saturated petroleum hydrocarbons [4,5]. This was confirmed by the presence of steranes and hopanes. These compounds were supposed to be responsible for the healing properties of Azerbaijani naphthalane preparations [6,7]. To the authors' knowledge, there was no clear evidence for NAN what was their amount in it.

NAN is composed of different terpenoids of varying structures (branching and cyclization), present in continuous homologous ranges of molecular masses, each appearing in a high isomer variety. Because of the extremely high number of constituents and the great similarity in their chemical and physical properties, their resolution is difficult. That classifies NAN as an ''unresolved complex mixture'' (UCM). For the analysis of steranes and hopanes, as focused NAN components, a HRGC (GC) and GC–MS coupled system was used, as the method of choice. In this complementary tool pair, GC served in order to provide a reasonable separation of NAN and to help quantification [by flame ionisation detection (FID)] and GC–MS was applied in order to identify steranes and hopanes and to quantify them on the basis of the relative intensities in the related specific mass fragmentograms. Both in GC and GC–MS analyses, non-polar columns were used (all NAN constituents are saturated). In GC–MS analyses, in order to make up for the loss of the component separation on the GC column caused by direct column coupling to the electron impact ion source (high vacuum), a comparatively longer column was applied.

## 2. Experimental

### 2.1. Sample

NAN is a non-aromatic [2], transparent, colourless and unctuous liquid composed of genuine petroleum compounds; it has density ($\rho$) at 15°C (ASTM D 1298) of $0.8819 \pm 0.0002$ g/cm$^3$ and refraction index ($n_D^{20}$) (ASTM D 1218) of $1.4811 \pm 0.0002$ and it is composed exclusively of carbon and of hydrogen ($C_{\%mass} = 86.5 \pm 0.3$ and $H_{\%mass} = 13.5 \pm 0.1$).

### 2.2. Conditions

#### 2.2.1. GC

A Carlo Erba 5300 MSCGC instrument and Supelco capillary column were used. Fused silics open tubular, non-polar, SPB-1, 30 m×0.25 mm, film thickness 0.25 μm, column temperature: start from 50°C, temperature increased by gradients of 10°C/min (in order to ensure a large range of hydrocarbons of different boiling points to perform reasonably well shaped elution zones/peaks), final temperature was 319°C (for 60 min); injection: temperature 300°C, splitting mode, 0.2 μl; carrier gas: helium 5.0, $t_0$ ($CH_4$): 90 s (at 50°C); detection: flame ionisation, temperature 319°C; calculating: by Varian Star 4.0, by normalisation, without response factors.

#### 2.2.2. GC–MS

GC instrument Varian 3700; capillary column J&W Scientific, FSCOT, non-polar, DB-1, 60 m× 0.248 mm, film thickness 0.25 μm, temperature: start from 100°C (to shorten the retention time and to improve the shape of the peaks eluting lately), temperature increased by gradient 10°C/min up to 200°C, than the temperature increase was followed by gradient 5°C/min (to improve the separation of the late eluting peaks) up to 319°C (for 30 min); injection as above; carrier gas as above; GC–MS coupling: direct inlet; MS instrument: Varian MAT 112S; scanning rate: 1 s/decade; interscan time: 0.2 s; ionisation: electron impact; electron energy: 70 eV; emission current: 0.7 mA; resolution: 1:600; $m/z$ range: 50–600; ion source temperature: 260°C; ion source pressure: $10^{-5}$ Pa.

Identification was performed with the help of the published fingerprints [5,8] of mass fragmentograms $m/z$ 217 and $m/z$ 218 for 14α(H) and 14β(H) steranes, respectively, and $m/z$ 191 for hopanes. Also, mass fragmentograms of the parent ions were used. Whenever needed, full mass spectra were scanned to support the identification by the help of published spectra [8] (no standard compounds were available). The relative amounts among the con-

stituents studied by GC–MS, were determined on the basis of peak height (in mass fragmentogram) ratios. Each compound type was measured in the related fragmentogram (i.e. the proportions among 14β(H) steranes were determined in fragmentogram $m/z$ 218 $m/z$ and among 14α(H) steranes in fragmentogram $m/z$ 217), in order to respect the fragmentation patterns and their reflection on the fragmentogram peak intensity. Diasteranes were calculated in fragmentogram $m/z$ 217.

## 3. Results and discussion

NAN performed a complex GC chromatogram composed of hundereds of peaks, (roughly) gathering into two humps (Fig. 1). In the later hump, a remarkable group of peaks appeared in a specific cluster. By scanning full mass spectra along the cluster, it was found to be prevailingly composed of steranes. The identification of the single sterane compounds was confirmed by mass fragmentograms, as shown in Fig. 2. When calculating the portion of GC chromatogram between the first eluting and the last eluting identified sterane; it was found that the monitored cluster made 33±1% of NAN. GC peaks (Fig. 1) α5 and the twin peak (β3+ β4) belonging to 14 α(H) 17α(H) 20R cholestane and 14β(H) 17β(H) 20R plus 14β(H) 17β(H) 20S ergostanes, respectively, were found to be reasonably clean (confirmed with full mass spectra) and to make 1.52±0.05% and 2.19±0.05%, respectively. By means of these data, concentrations of the identified 14α(H) steranes and 14β(H) steranes were estimated in mass fragmentograms $m/z$ 217 and $m/z$ 218, respectively. In spite of approximations in calculating, the results in Table 1 (the labels are the same as the related peak labels in Figs. 1 and 2) offer a reasonable insight in the distribution of steranes in NAN.

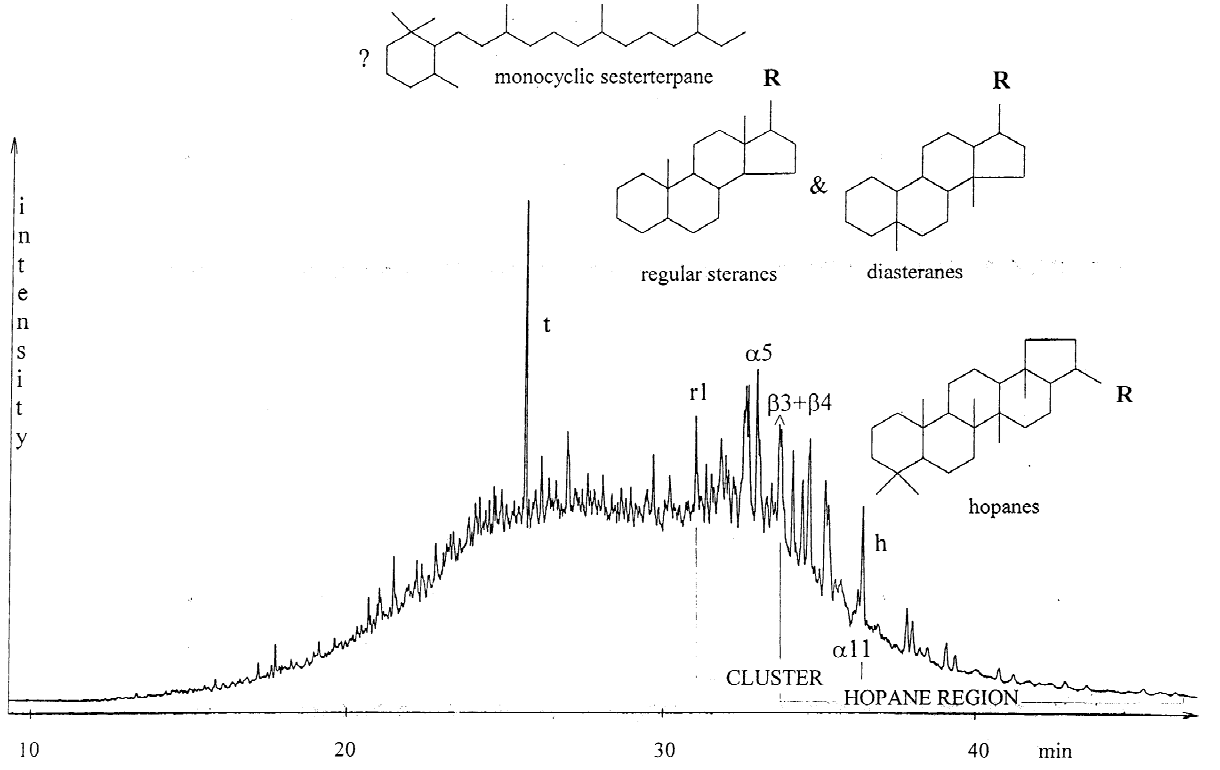


Fig. 1. GC chromatogram of NAN. Fused silica open tubular column; SPB-1; 30 m×0.25 mm; film thickness: 0.25 μm, temperature: 50°C, 10°C/min; 319°C for 60 min; injection: splitting 0.2 μl; $t$ ($CH_4$): 90 s.

a)

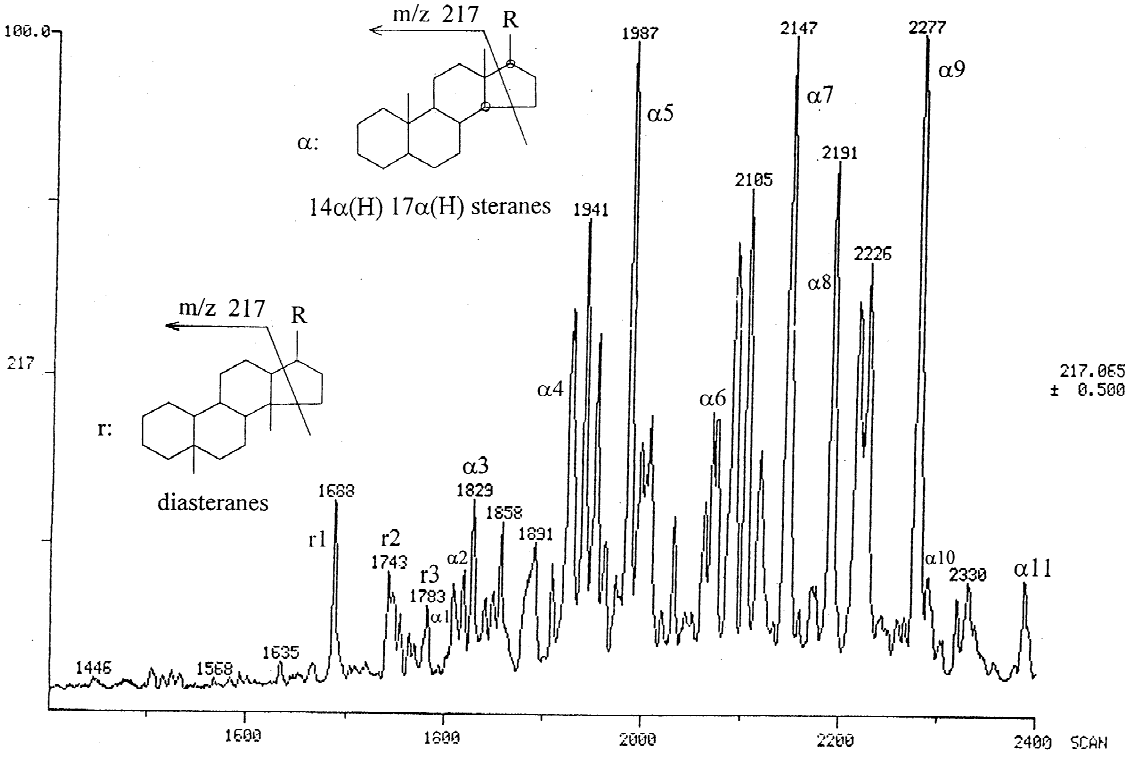


Fig. 2. Mass fragmentograms: (a) $m/z$ 217 and (b) $m/z$ 218. Peak labels are the same as in the Table 1. GC: fused silica open tubular; DB-1; 60 m×0.248 mm; film thickness: 0.25 μm; temperature program start: 100°C, 10°C/min to 200°C, 5°C/min to 319°C; GC–MS coupling: direct inlet; MS: scanning rate 1 s/decade; interscan time: 0.2 s; ionisation electron impact; electron energy 70 eV; emission current 0.7 mA; resolution 1:600; $m/z$ range: 50–600; ion source temperature: 260°C; ion source pressure $10^{-5}$Pa.

The identified and quantified steranes were in the range starting from $C_{26}H_{46}$ up to $C_{30}H_{54}$, making 16.2% of NAN together. Among them, 14α(H)-steranes were more intense (8.9%) than 14β(H)-steranes (6.4%). Regular ones were found to be 17 times more abundant than (identified) diasteranes (0.9%). A massive portion of the steranes belonged to regular cholestanes ($C_{27}H_{48}$) (4.3%), ergostanes (4.5%) and stigmastanes (4.7%), which made 13.5% of NAN together.

Steranes determined in NAN were found present in a stereo variety mainly related to the hydrogen configuration in positions 14, 17 and 20 (as ordinary petroleum steranes). The limited number of sterane stereo-isomers (regarding the number of asymmetric centres) in NAN may be explained [5] by biologic origin of sterane precursors (stereo-chemistry limited and strictly controlled), as well as by the geochemistry rules of their geogenic transformation [tendency to equilibrium of 14α(H) 17α(H): 14β(H) 17β(H), and 20R: 20S configurations, what observable by an ordinary GC tool]. Diasteranes appearance in NAN (r1 -r3 in Fig. 2 and Table 1) can be explicable [5] by the rearrangement of the angular methyl groups from the positions 10 and 13 to the positions 5 and 14 in geological environment.

Steranes in NAN remained structurally related to their bio-precursors and they were analogous in their skeleton to bioactive steroids, among which there are sex and adrenal cortex hormones, bile acids, heart toxins, as well as pro-vitamins D. For example, ergosteranes have a skeleton as ergosterol and as

b)

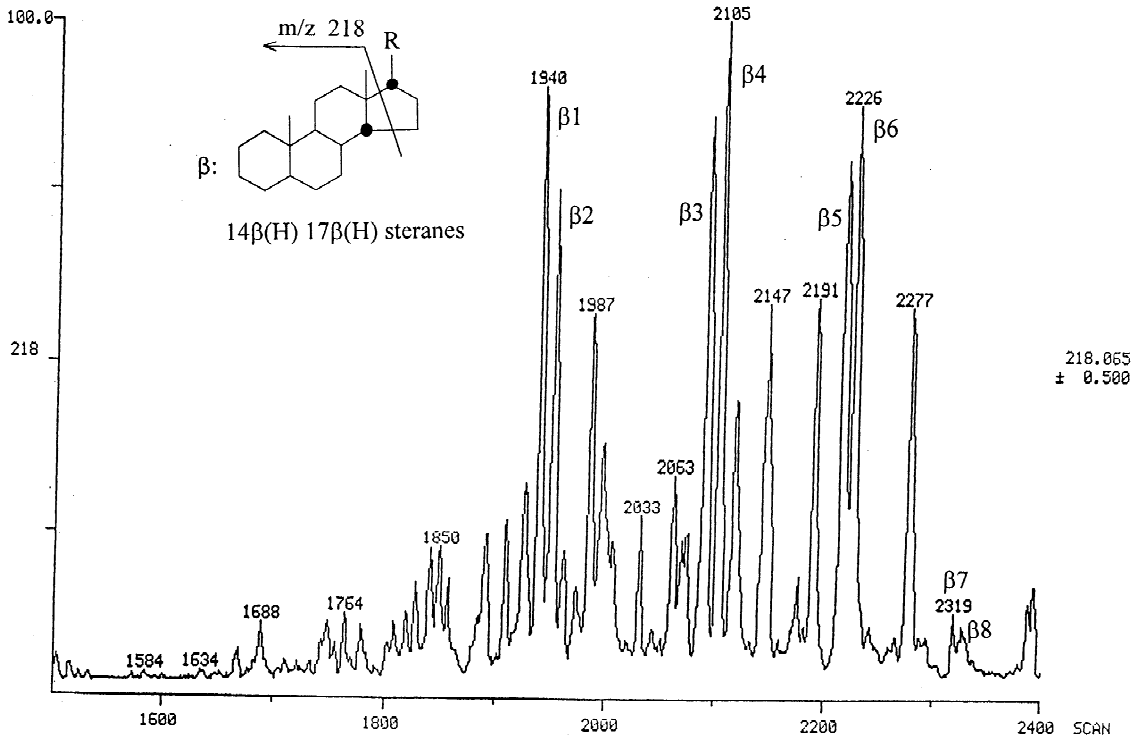


Fig. 2. (*continued*)

$C_{22}$–$C_{23}$ dihydroergosterol, which by UV irradiation turn to ergocalciferol (vitamin $D_2$; vitamin $D_1$ = ergocalciferol + lumisterol) and dihydroergocalciferol (vitamin $D_4$), respectively. Also, cholestanes may be taken as analogues to 7-dehydrocholesterol which is the precursor of cholecalciferol, i.e. vitamin $D_3$. Cholestanes have a skeleton analogous to cholesterol which, among others, serves as a precursor for the preparation of testosterone. Also, the cholestane derivative cholestanyl acetate can be used in the synthesis of androsterone, as well as stigmasteryl acetate, cholesterol and ergosterol for progesterone. Among others, there are skeleton similarities between steranes and corticosteroids.

The physiological equivalency of bacteriohopantetraol in prokaryotes to steroids in eukaryotes, as well as the pentacyclic structure character, made hopanes to be intriguing constituents of NAN. Among them, shown in a GC chromatogram 17α(H) 21β(H) hopane (h5 in Table 1, i.e., h in Fig. 1) and the series of 17α(H) 21β(H) homohopane homologues appearing in resolved pairs of 20*R* and 20*S* were recognised. According to the amount of a relatively well resolved GC peak h (confirmed by full mass spectrum), which made up 1.17±0.05% of NAN (Table 2) and according to the mass fragmentogram *m*/*z* 191 (Fig. 3), the determined hopanes together were 4.7% of NAN. The amount of identified norhopanes, interfering with the sterane cluster was found to be less than 1% of NAN.

The prominent peak (t in Fig. 1) seemed to be a sesterterpane and made up 2.16±0.05% of NAN. It should be submitted to a thorough study in order to be fully identified.

Among the cyclic terpenoids, earlier studied in Croatian petroleum [9], components were indicated as rather possibly comprising the ionone ring. The side chains in petroleum constituents are known to

Table 1
Steranes in non-aromatic Croatian naphthalane (NAN)

| No. | $M^+$ | Btto form. | Sterane | % |
|---|---|---|---|---|
| 14α(H)-Steranes (determined from mass fragmentogram *m/z* 217, Fig. 2a) | | | | |
| α1 | 358 | $C_{26}H_{46}$ | 14α(H) 17α(H) 21-norcholestane | 0.22 |
| α2 | 358 | $C_{26}H_{46}$ | 14α(H) 17α(H) norcholestane 20S | 0.27 |
| α3 | 358 | $C_{26}H_{46}$ | 14α(H) 17α(H) 27-norcholestane 20S | 0.50 |
| α4 | 372 | $C_{27}H_{48}$ | 14α(H) 17α(H) cholestane 20S | 0.89 |
| α5[a] | 372 | $C_{27}H_{48}$ | 14α(H) 17α(H) cholestane 20R | 1.52 |
| α6 | 386 | $C_{28}H_{50}$ | 14α(H) 17α(H) ergostane 20S | 0.72 |
| α7 | 386 | $C_{28}H_{50}$ | 14α(H) 17α(H) ergostane 20R | 1.54 |
| α8 | 400 | $C_{29}H_{52}$ | 14α(H) 17α(H) stigmastane 20S | 1.22 |
| α9 | 400 | $C_{29}H_{52}$ | 14α(H) 17α(H) stigmastane 20R | 1.54 |
| α10 | 414 | $C_{30}H_{54}$ | 14α(H) 17α(H) *n*-propyl cholestane 20S | 0.23 |
| α11 | 414 | $C_{30}H_{54}$ | 14α(H) 17α(H) *n*-propyl cholestane 20R | 0.24 |
| | | | | Σ8.89 |
| 14β(H)-Steranes (determined from mass fragmentogram *m/z* 218, Fig. 2b) | | | | |
| β1 | 372 | $C_{27}H_{48}$ | 14β(H) 17β(H) cholestane 20R | 1.05 |
| β2 | 372 | $C_{27}H_{48}$ | 14β(H) 17β(H) cholestane 20S | 0.86 |
| β3[a] | 386 | $C_{28}H_{50}$ | 14β(H) 17β(H) ergostane 20R | 1.03 |
| β4[a] | 386 | $C_{28}H_{50}$ | 14β (H) 17β(H) ergostane 20S | 1.16 |
| β5 | 400 | $C_{29}H_{52}$ | 14β(H) 17β(H) stigmastane 20R | 0.92 |
| β6 | 400 | $C_{29}H_{52}$ | 14β(H) 17β(H) stigmastane 20S | 1.02 |
| β7 | 414 | $C_{30}H_{54}$ | 14β(H) 17β(H) *n*-propyl cholestane 20R | 0.15 |
| β8 | 414 | $C_{30}H_{54}$ | 14β(H) 17β(H) *n*-propyl cholestane 20S | 0.22 |
| | | | | Σ 6.41 |
| Rearranged steranes (determined from mass fragmentogram *m/z* 217, Fig. 2a) | | | | |
| r1 | 358 | $C_{26}H_{46}$ | dia-27-norcholestane | 0.44 |
| r2 | 372 | $C_{27}H_{48}$ | diacholestane | 0.27 |
| r3 | 372 | $C_{27}H_{48}$ | diacholestane | 0.21 |
| | | | | Σ 0.91 |
| | | | | ΣΣ 16.21 |

[a] Used in calculation.

occur frequently in the isoprenoidal form [5]. These compounds deserve to be studied since they are similar to the skeleton of vitamin A.

## 4. Conclusion

Hoping to bring some elucidation in the complexity of the subject related to understanding the influence of NAN composition on its healing efficacy, steranes and hopanes were analysed. The combination of the GC and GC–MS methods enabled the determination of these focused NAN components regardless of being overlapped with other constituents, and even when in relatively low percentages, in a rather complex mixture of hydrocarbons such as NAN.

The GC cluster prevailingly composed of sterane peaks (in the range from norcholestanes to propyl cholestanes), made one third of NAN. Half of the cluster was composed of the identified steranes. Among them, regular cholestanes, ergostanes and stigmastanes, contributed 83%. The stereo-isomerization was reflected in positions 14, 17 and 20 and the highest concentration pertained to steranes, which spared the biological configuration. The rearranged steranes were low.

In the skeleton, steranes remained analogous to the natural and synthesised steroid bioactive compounds, among which there are vitamins, hormones and medicaments.

Table 2
Hopanes in non-aromatic Croatian naphthalane (NAN)[a]

| No. | $M^+$ | Btto form. | Hopane | % |
|---|---|---|---|---|
| h1 | 370 | $C_{27}$ $H_{46}$ | 18α(H) 22, 29, 30-trisnorneohopane | 0.12 |
| h2 | 370 | $C_{27}$ $H_{46}$ | 17α(H) 22, 29, 30-trisnorhopane | 0.18 |
| h3 | 398 | $C_{29}$ $H_{50}$ | 17α(H) 21β(H) 30-norhopane | 0.49 |
| h4 | 398 | $C_{29}$ $H_{50}$ | 17β(H) 21α(H) 30-normoretane | 0.09 |
| h5[b] | 412 | $C_{30}$ $H_{52}$ | 17α(H) 21β(H) hopane | 1.17 |
| h6 | 412 | $C_{30}$ $H_{52}$ | 17β(H) 21α(H) moretane | 0.23 |
| h7 | 426 | $C_{31}$ $H_{54}$ | 17α(H) 21β(H) 29-homohopane 22S | 0.44 |
| h8 | 426 | $C_{31}$ $H_{54}$ | 17α(H) 21β(H) 29-homohopane 22R | 0.39 |
| h9 | 426 | $C_{31}$ $H_{54}$ | 17β(H) 21α(H) 29-homomoretane 22S +22R | 0.13 |
| h10 | 440 | $C_{32}$ $H_{56}$ | 17α(H) 21β(H) 29-bishomohopane 22S | 0.34 |
| h11 | 440 | $C_{32}$ $H_{56}$ | 17α(H) 21β(H) 29-bishomohopane 22R | 0.28 |
| h12 | 454 | $C_{33}$ $H_{58}$ | 17α(H) 21β(H) 29-trishomohopane 22S | 0.26 |
| h13 | 454 | $C_{33}$ $H_{58}$ | 17α(H) 21β(H) 29-trishomohopane 22R | 0.18 |
| h14 | 468 | $C_{34}$ $H_{60}$ | 17α(H) 21β(H) 29-tetrakishomohopane 22S | 0.16 |
| h15 | 468 | $C_{34}$ $H_{60}$ | 17α(H) 21β(H) 29-tetrakishomohopane 22R | 0.10 |
| h16 | 482 | $C_{35}$ $H_{62}$ | 17α(H) 21β(H) 29-pentakishomohopane 22S | 0.09 |
| h17 | 482 | $C_{35}$ $H_{62}$ | 17α(H) 21β(H) 29-pentakishomohopane 22R | 0.05 |
| | | | | Σ4.70 |

[a] Determined from mass fragmentogram *m*/*z* 191, Fig. 3.
[b] Used in calculation.

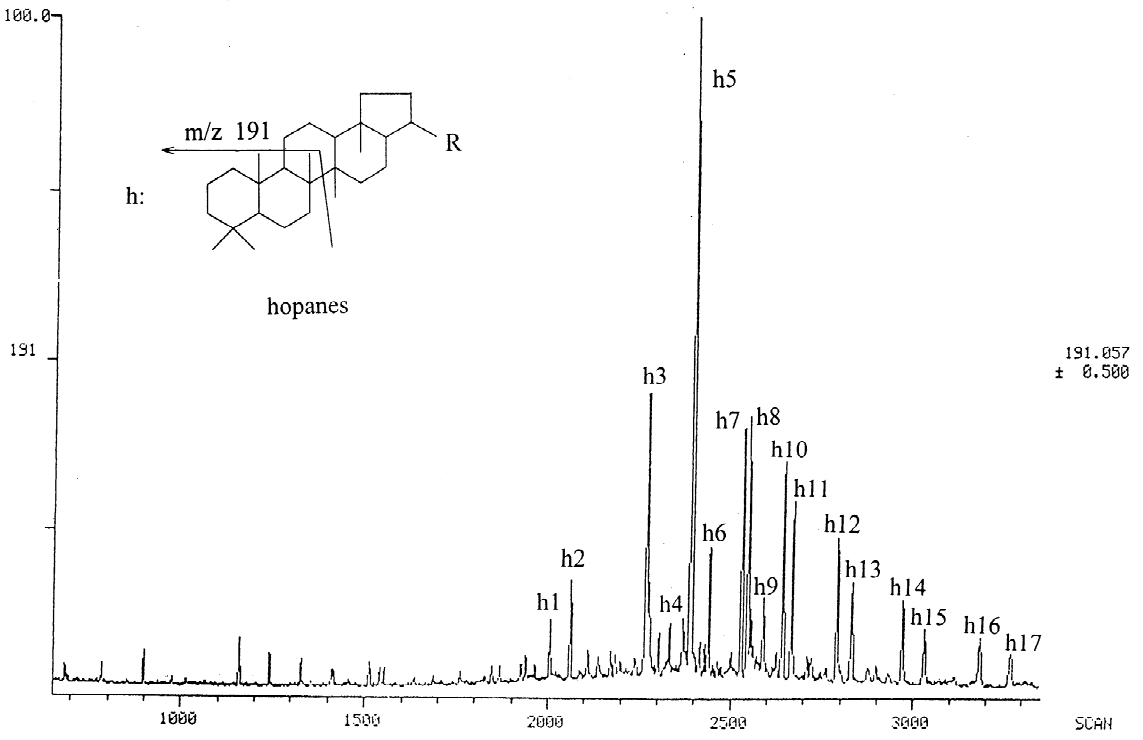


Fig. 3. Mass fragmetogram *m*/*z* 191. Peak labels are the same as in the Table 2. (Working conditions as for Fig. 2.)

Hopanes significantly contributed to the common content of oligocyclic hydrocarbons in NAN.

In spite of the fact that small differences in structure can cause great differences in the bioactivity direction and/or potency, demanding that all structural similarities and differences should be considered cautiously, the observed skeleton analogies are to be taken into account. On the way to finding the answer to what makes NAN bioactive, the focused (groups of) compounds deserve to be isolated and subjected to biomedical research in order to establish whether they are the constituents responsible for the curing effect of NAN.

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