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FLAVONOID PROFILES OF NEW ZEALAND KAURI AND OTHER SPECIES OF *AGATHIS*

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Key Word Index—*Agathis australis*; Araucariaceae; chemotaxonomy; flavonoids; biflavonoids; cupressuflavone; agathisflavone.

Abstract—This paper describes the flavonoid constituents in *Agathis australis* from a wide geographic range and completes a comprehensive survey of the flavonoid chemistry/chemotaxonomy of New Zealand conifers begun in 1984. Flavonol 3-*O*-glycosides and biflavones of the cupressuflavone and agathisflavone types are shown to be the predominant flavonoids. NMR data for these biflavones with varying degrees of methylation are presented for the first time. The flavonoid profiles of *A. australis* and three other species of *Agathis* show a high level of uniformity, and in this respect are closely related.

INTRODUCTION

The genera *Agathis* Salisb. and *Araucaria* Juss. belong to the Southern Hemisphere conifer family Araucariaceae Henkel & W. Hochst [1]. *Agathis australis* (D. Don) Lindl, the familiar and endemic kauri, is the sole representative of the family in New Zealand. It is also the only temperate member of an otherwise tropical genus of about 13 species extending northwards to Melanesia and Malesia [2].

Agathis australis is confined to the northern parts of New Zealand [3], and appears to be a uniform taxon throughout its natural range. *Agathis* also has been considered an easily circumscribed genus of related species, not forming marked subgeneric groups [4], although some taxonomists have seen *A. australis* as occupying an isolated position within this genus [5]. Others [2, 6] have distanced *A. australis* from some members of the genus while aligning it with others.

The survey of flavonoid constituents in *A. australis* reported here was undertaken in order (a) to determine the flavonoid profiles of *A. australis* and other species of *Agathis* for taxonomic purposes and (b) to complete the comprehensive survey of the flavonoid chemistry/chemotaxonomy of New Zealand conifers begun in 1984 [7–16]. Previous studies of the flavonoids in *Agathis* have been confined to the biflavonoid constituents. Agathisflavone, cupressuflavone, amentoflavone and robustaflavone have all been reported previously, together with a variety of their methyl ethers and a mono-methyl ether of hinokiflavone [17]. However, the level of support for claimed structures is variable, and for a number, sound NMR data have not yet appeared in the literature.

RESULTS AND DISCUSSION

Biflavonoid constituents

The biflavonoids in *A. australis* were isolated from the dried EtOH–H_2O extract using methyl ethyl ketone extraction followed by column and/or preparative thin-layer chromatography. Apigenin-based biflavones of the cupressuflavone (8–8″ linked) and agathisflavone type (6–8″ linked) were present, in quantity, methylated at the 4′, 4‴, 7 and 7″ positions and combinations thereof. These compounds were identified by ^1H and/or ^{13}C NMR spectroscopy with relevant connectivities confirmed by ^1H–^1H and ^1H–^{13}C COSY spectra, except for agathisflavone 4′,4‴,7,7″-tetramethyl ether, which was identified by TLC only.

For the compound identified as cupressuflavone 7,7″-dimethyl ether, an examination of its mass spectrum [LSIMS (+)NBA matrix] revealed a molecular ion at 567 amu. This suggested the compound was a biapigenin containing two methoxyl substituents. The ^{13}C NMR data supported this, and the appearance of only 14 signals was suggestive of a symmetrical biflavonoid. Oxygenation at C-4′ in both B-rings was evident from both the ^1H and ^{13}C NMR spectra, the former showing characteristic *ortho*-coupled doublets for the H-2′6′ and H-3′5′ protons at $\delta 7.46$ and $\delta 6.77$, respectively. The fact that the A ring proton signals did not show any coupling indicated that the linkage was between the A rings, and the appearance of the C-8, C-8″ resonance at a position

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5 ppm (δ99.0) downfield relative to that of the C-8 of the monomeric unit, apigenin (δ94.0), indicated that the linkage was of the cupressuflavone type (8′–8″). The methoxy substitution was established to be at C-7 and C-7″ on the basis of, firstly, the UV spectral shifts induced by AlCl$_3$/HCl and NaOMe, which revealed the presence of a free 5-OH and 4′-OH, respectively, and secondly, the presence of a signal in the ^{1}H NMR for H-6 adjacent to a 7′-OH (e.g. apigenin δ6.25 [18]).

The 7-,4′,7,7″- and 4′,4‴,7,7″-*O*-methyl ethers of cupressuflavone were all identified in a similar way from their mass spectra and their ^{1}H and ^{13}C NMR spectra. For example, the 7-*O*-methyl ether exhibited only one three-proton methoxyl signal, and the H-6 and 6″ signals appeared as two distinct singlets. One of these had shifted upfield to δ6.45 relative to its shift in cupressuflavone 7,7″-dimethyl ether, consistent with the presence of a hydroxyl at the 7″ position.

With the compound identified as cupressuflavone 4′,7,7″-trimethyl ether, an additional methoxy signal was observed, and the H-3′5′ and H-3‴5‴ signals occurred as two distinct doublets. One doublet at δ6.76 had a shift very similar to that seen for the superimposed H-3′5′, H-3‴5‴ doublet of the 7,7″-dimethyl ether, whilst the other was shifted downfield to δ6.98 suggesting that the location of the additional methoxy substituent was C-4′.

The mass spectrum for the compound identified as agathisflavone 7,7″-dimethyl ether was similar to that of cupressuflavone 7,7″-dimethyl ether, in that it exhibited a parent ion at 566 amu. However, the ^{1}H NMR spectrum showed a doubling in the number of signals compared with cupressuflavone 7,7″-dimethyl ether, thereby revealing that the compound was a similar but non-symmetrical biflavonoid. Significantly, the A-ring protons showed no coupling, and this fact combined with the requirement that the molecule be non-symmetrical, meant that the interflavonoid linkage must be of the agathisflavone type (6-8″). Oxygenation at the 4′- and 4‴-positions in the B-rings was established from the ^{1}H NMR spectrum, which revealed two sets of *ortho*-coupled doublets at δ8.04/7.53 and δ6.98/6.77, and methoxyl groups were sited at C-7 and 7″ in an analogous way to that used for the assignments in the cupressuflavone equivalent.

Agathisflavone 7-*O*-methyl ether was determined to be a mono-*O*-methyl derivative from its ^{1}H NMR spectrum. The methoxyl was sited at C-7 since, as for the cupressuflavone equivalent, the H-6″ was shifted upfield relative to its position in the spectrum of the 7,7″-dimethyl ether. In a like manner, agathisflavone 4′,7,7″-trimethyl ether was also identified by comparison of its ^{1}H NMR sepctrum with that of the 7,7′-dimethyl ether. Thus, apart from the

Table 1. ^{1}H NMR data for *Agathis* biflavonoids (in DMSO-d_6)

Name	I II	2 2″	3 3″	5 5″	6 6″	7 7″	8 8″	2′ 2‴	3′ 3‴	4′ 4‴	5′ 5‴	6′ 6‴
Cupressuflavone 7-*O*-methyl ether	I	— —	6.82 *s*		6.74 *s*	3.80 *s* OMe	C |	7.51 *d* (8.8)	6.75 *d* (8.7)		6.75 *d* (8.7)	7.51 *d* (8.8)
	II		6.78 *s*		6.45 *s*		C	7.43 *d* (8.8)	6.75 *d* (8.7)		6.75 *d* (8.7)	7.43 *d* (8.8)
Agathisflavone 7-*O*-methyl ether	I	—	6.93 *s*		C-	3.82 OMe	7.04 *s* |	8.04 *d* (8.6)	6.98 *d* (8.6)		6.98 *d* (8.6)	8.04 *d* (8.6)
	II		6.76 *s*		6.35 *s*		 C-	7.51 *d* (8.8)	6.75 *d* (8.6)		6.75 *d* (8.6)	7.51 *d* (8.8)
Cupressuflavone 7,7″-dimethyl ether	I	—	6.86 *s*	OH 13.32	6.78 *s*	3.82 *s* OMe	C |	7.46 *d* (8.6)	6.77 *d* (8.6)		6.77 *d* (8.6)	7.46 *d* (8.6)
	II		6.86 *s*	OH 13.22	6.78 *s*	3.82 *s*	C	7.46 *d* (8.6)	6.77 *d* (8.6)		6.77 *d* (8.6)	7.46 *d* (8.6)
Agathisflavone 7,7″-dimethyl ether	I	—	6.93 *s*		C-	3.83 *s* OMe	7.05 *s*	8.04 *d* (8.7)	6.98 *d* (8.7)		6.98 *d* (8.7)	8.04 *d* (8.7)
	II		6.82 *s*		6.64 *s*	3.82 *s*	 C-	7.53 *d* (8.7)	6.77 *d* (8.7)		6.77 *d* (8.7)	7.53 *d* (8.7)
Cupressuflavone 4′,7,7″-trimethyl ether	I	—	6.95 *s*		6.75 *s*	3.804 *s* OMe	C |	7.57 *d* (9)	6.98 *d* (9)	3.789	6.98 *d* (9)	7.57 (9)
	II		6.86 *s*		6.77 *s*	3.796 *s*	C	7.45 *d* (8.5)	6.76 *d* (8.6)	OMe	6.76 *d* (8.6)	7.45 *d* (8.5)
Agathisflavone 4′,7,7″-trimethyl ether	I	—	6.91 *s*		C-	3.82 *s* OMe	7.05	8.04 *d* (8.9)	6.97 *d* (8.7)	3.77	6.97 (8.7)	8.04 *d* (8.9)
	II		6.83 *s*		6.65 *s*	3.81 *s*	 C-	7.63 *d* (8.9)	6.97 (8.7)	OMe	6.97 (8.7)	7.63 *d* (8.9)
Cupressuflavone 4′,4‴,7,7″-tetra methyl ether	I	—	6.97 *s*		6.78 *s*	3.81 *s* OMe	C |	7.59 *d* (8.8)	7.03 *d* (9.0)	3.79	7.03 *d* (9.0)	7.59 *d* (8.8)
	II		6.97 *s*		6.78 *s*	3.80 *s*	C	7.59 *d* (8.8)	7.03 *d* (9.0)	OMe	7.03 *d* (9.0)	7.59 *d* (8.8)

presence of three methoxyl signals, the only significant difference relative to the spectrum of the 7,7″-dimethyl ether was the downfield shift of the H-3‴5‴ doublet to δ6.97. This suggests that the location of the additional methoxyl group is at C-4‴.

A comprehensive presentation of the above NMR data is made for the first time (see Tables 1 and 2), although some were included without discussion in a recent compilation of flavonoid ^{1}H NMR spectra [18].

The phenomenon of optical activity of biflavones is an interesting one in that a number of derivatives of biflavones of the 8–8″-linked cupressuflavone type have been shown to be optically active [19–21], and others not [22]. The cupressuflavone 7,7″-di-*O*-methyl ether isolated in the course of this work was shown to be optically active ($[\alpha]_D^{20^\circ} = +63.3^\circ$) ($Me_2CO\{1.12\}$) but the 6–8″-linked agathisflavone 7,7″-di-*O*-methyl ether was apparently optically inactive ($[\alpha]_D^{20^\circ} \approx 0^\circ$) ($Me_2CO$ {0.11}). This is surprising since the number and type of *O*-substituent interactions that give rise to restricted rotation about the interflavonoid bond are similar in both, and some derivatives of agathisflavone have already been shown to be optically active [7, 23]. A possible explanation may be that the production of agathisflavone derivatives in this plant may be via a non-enzymic (Wessely–Moser type) rearrangement of an 8,8″-linked biosynthetic precursor. Alternatively, racemization may occur preferentially during work-up of the agathisflavone derivative if the energy of rotation is less than for the equivalent cupressuflavone.

Flavonoid O-*glycoside constituents*

Flavonoid *O*-glycoside profiles of *A. australis* were determined by 2D-PC, with comparison in UV light before and after separate treatment with NH_3 and NA spray reagent. Individual components were isolated from paper chromatograms or by column chromatography and identified by standard techniques [24], including ^{1}H NMR and co-chromatography with authentic standards. The major component was identified as rutin and its inter-glycosidic linkage was confirmed by ^{1}H NMR spectroscopy [24]. Other flavonoids, generally isolated in small quantities by column chromatography, were identified as kaempferol-3-rhamnoside and -3-rutinoside, quercetin-3-rhamnoside and -3-glucoside, 7-methylquercetin-5-glucoside, vitexin, isovitexin, apigenin-7-glucoside, catechin and epicatechin. Apart from kaempferol- and quercetin-3-rutinosides and quercetin-3-glucoside, these glycosides were rarely discernable on the 2D-PCs at standard loading. The main variable within the *A. australis* range studied was the level of quercetin-3-glucoside which ranged from dominant in the Great Barrier Island sample to barely evident in the Atuanui and Kaiaka samples. Otherwise, the patterns of flavonoid glycosides were remarkably similar.

Survey of other species of Agathis

Samples of non-New Zealand species of *Agathis* representing three of the species groups of Whitmore [2]

Table 2. ^{13}C NMR data for *Agathis* biflavonoids (in DMSO-d_6)

Name	I	2	3	4	5	6	7	8	9	10	1′	2′	3′	4′	5′	6′	OMe
	II	2″	3″	4″	5″	6″	7″	8″	9″	10″	1‴	2‴	3‴	4‴	5‴	6‴	OMe
Cupressuflavone	I	163.3	102.5	182.3	161.7	99.5	164.0	95.4	154.7	114.3	121.1	128.1	115.9	161.1	115.9	128.1	56.4
7-*O*-methyl ether	II	163.2	102.5	181.9	161.3	98.6	163.2	98.1	153.8	103.5	121.1	127.7	115.9	160.9	115.9	127.7	
Agathisflavone	I	164.0	103.1	181.9	158.7	95.4	164.4	90.7	157.1	104.6	121.2	128.6	116.0	161.6	116.0	128.5	56.4
7-*O*-methyl ether	II	162.6	102.5	181.9	157.2	98.6	160.6	92.7	154.6	103.7	121.0	127.8	116.0	161.0	116.0	127.8	
Cupressuflavone	I	163.0	102.5	182.2	161.7	95.5	163.8	98.8	153.8	104.2	120.9	127.9	116.0	161.8	116.0	127.9	56.5
7,7′-dimethyl ether	II	161.2	102.5	182.2	161.7	95.5	163.8	99.0	153.8	104.2	120.9	127.9	116.0	161.8	116.0	127.9	
Agathisflavone	I	163.8	103.1	182.2	158.6	103.5	164.1	90.8	153.7	104.6	121.1	128.6	116.0	161.5	116.0	128.6	56.4
7,7″-dimethyl ether	II	163.8	102.5	182.0	157.2	95.5	163.2	99.8	153.7	104.1	121.0	128.0	115.6	161.4	116.0	128.0	56.4
Cupressuflavone	I	162.4	102.3	181.2	160.8	94.5	162.1		152.9	103.3	113.6	126.7	113.6	161.3	113.6	126.7	55.4
4,4‴,7,7″-tetramethyl ether	II	162.4	102.3	181.2	160.8	94.5	162.1		152.9	103.3	113.6	126.7	113.6	161.3	113.6	126.7	54.5

Table 3. Survey of biflavonoid types in *Agathis* species

Name	*A. australis*	*A. ovata*	*A. atropurpurea*	*A. robusta*
Cupressuflavone 4′,4‴,7,7″-tetramethyl ether	·	·	×	×
Agathisflavone 4′,4‴,7,7″-tetramethyl ether	·	·	×	×
Cupressuflavone 4′,7,7″-trimethyl ether	·	·	·	×
Agathisflavone 4′,7,7″-trimethyl ether	·	·	·	×
Cupressuflavone 7,7″-dimethyl ether	○	○	o	o
Agathisflavone 7,7″-dimethyl ether	o	o	○	○
Cupressuflavone 7-*O*-methyl ether	·	·	o	o
Agathisflavone 7-*O*-methyl ether	·	·	o	○

○ Major.
o ↓.
· Minor.
× Not detected.

and the leaf groups of Page [6] were surveyed and their flavonoid profiles compared to that of *A. australis*. The samples used and their sources are given below. Flavonoid glycosides were surveyed by 2D-PC and TLC and visualized with NA spray. Less polar flavonoids were surveyed by TLC and visualized with diazotized sulphanilic acid spray (Table 3).

With respect to the biflavonoid constituents, *A. ovata* was found to be similar to *A. australis* both qualitatively and quantitatively, being possibly distinguished only by an increased accumulation of agathisflavone 7,7″-dimethyl ether. In *A. atropurpurea* the less methylated biflavones predominated, whereas neither the highly methylated cupressuflavone 4′,4‴,7,7″-tetramethyl ether nor agathisflavone 4′,4‴,7,7″-tetramethyl ether were detected. This tendency to accumulate the less methylated biflavones was even more marked in *A. robusta*, where agathisflavone 7-methyl ether was a major component, but neither the trimethyl ethers nor the tetramethyl ethers of agathisflavone or cupressuflavone were found.

The flavonoid glycosides were equally indistinct. Thus, varying mixes of the *A. australis* flavonoid glycosides were found in all the other species of *Agathis* and no new compounds were detected. Only one species, *A. atropurpurea*, possessed a distinctive profile, with the monoglycosides quercetin-3-rhamnoside and -3-glucoside being equally as prominent as the rutinoside. A trace of kaempferol-3-rhamnoside was also evident. Quercetin-3-rhamnoside is otherwise a significant flavonoid only in the profile of *A. australis* from Great Barrier Island. In general however, it is concluded that these species of *Agathis* cannot be distinguished with confidence from one another, or from *A. australis*, on the basis of their flavonoid *O*-glycoside profiles alone.

Dominance of flavonol glycosides of the type found in *Agathis* is unusual amongst the New Zealand conifers. Our earlier surveys of *Podocarpus*, *Prumnopitys*, *Dacrycarpus*, *Phyllocladus*, *Libocedrus*, *Dacrydium*, *Lepidothamnus*, *Halocarpus* and *Lagarostrobos* indicate that only in *Prumnopitys* are such glycosides characteristic of the genus and accumulated in quantity [9].

On the basis of the biflavonoid and flavonoid glycoside distributions evaluated here, it is concluded that *Agathis*, from the regions sampled, is a remarkably uniform genus. *Agathis australis* from New Zealand is not convincingly distinguished from the three other species studied by its flavonoid profile, suggesting that it may not occupy the isolated position proposed by some taxonomists [2, 5, 6]. These results lend support to the view, based on conventional character-states, that *Agathis* is a genus of closely related species with only subtle differences between them [4].

EXPERIMENTAL

Plant material, sources and herbarium numbers. Agathis australis (D. Don) Lindl.: Christchurch Botanic Gardens (CHR 418728); cult, ex Waipoua, growing in Landcare Research Experimental Gardens, Lincoln (CHR 474630); Kaiaka, Northland (CHR 291138—sampled ex herb. specimen); Atuanui, Northland (CHR 195382—sampled ex herb. specimen); Great Barrier Is., Auckland (CHR 232977—sampled ex herb. specimen); Katikati, Bay of Plenty (CHR 213488—sampled ex herb. specimen). *Agathis robusta* (F. Muell.) F. M. Bailey: Clohesy R., Queensland (CHR 437636) and Atherton Arboretum, Queensland (CHR 437637). *Agathis atropurpurea* B. Hyland: Atherton Arboretum, Queensland (CHR 437629A). *Agathis ovata* (Vieil. Warb.): Montagne des Sources, New Caledonia (CHR 437611).

Agathis australis *extraction and work-up.* Dry leaves (CHR 418728) (170 g) were powdered and extracted × 3 with EtOH:H_2O, 1:1. The combined extracts were reduced to dryness. MEK was added to the residue, and the MEK solubles were extracted with H_2O. CC of the MEK solubles (polyamide SC6) using Me_2CO–MeOH–H_2O, 2:1:2 as the eluent gave a series of frs. Final purification of the biflavonoids was achieved on Sephadex LH20 (Me_2CO–MeOH–H_2O, 2:1:1) or by prep. silica TLC (toluene–Me_2CO–$CHCl_3$, 8:7:5).

Amberlite XAD-7 beads were added to the flavonoid glycoside containing H_2O fr. from above, and allowed to soak overnight. The brown beads were then added to the top of an Amberlite XAD7 column. The column was washed with H_2O (1.5 l) and eluted, first with $MeOH-H_2O$, 1:1, and then with 100% MeOH. Both eluates were reduced in volume and combined. The mixture was further fractionated by DCCC using *n*-BuOH-MeOH-H_2O, 5:1:5. The *n*-BuOH layer served as the stationary phase and the aqueous layer as the mobile phase (ascending mode). Separation of the resulting frs was achieved by prep. polyamide TLC using $Me_2CO-MeOH-H_2O$, 2:1:1 or by PC (15% HOAc). Final purification was by CC on Sephadex LH20 with $H_2O-MeOH-Me_2CO$, 3:1:1.

In an alternative work-up the $EtOH-H_2O$ extract was chromatographed on a polyamide (MN SC-6) column in H_2O with increasing amounts of MeOH to 100% MeOH. Elution was continued by gradually replacing MeOH with Me_2CO up to 100% Me_2CO. Analysis and further purification was carried out by TLC and 2D-PC, respectively. The catechins and glycosides were found in the $MeOH-H_2O$ frs and the biflavonoids in the Me_2CO containing frs.

General methods for TLC and 2D-PC surveys of A. australis *and other species of* Agathis. Dry leaves (*ca* 2 g) were extracted with $EtOH-H_2O$, 1:1. Each extract was passed through polyamide powder to remove tannins, reduced in volume and then made up to 50 ml with EtOH. A portion of each extract (0.8 ml) was evaporated to dryness. The residue was partitioned between MEK and H_2O. The MEK frs were analysed by silica TLC in toluene-pyridine-HCO_2H, 36:9:5, toluene-$CHCl_3$-Me_2CO, 8:5:7 and by polyamide TLC in $Me_2CO-MeOH-H_2O$, 1:1:1 and visualized with diazotized sulphanilic acid spray. The H_2O frs were analysed for flavonoid glycosides by silica TLC in EtOAc-MEK-HCO_2H-H_2O, 5:3:1:1, and PC-cellulose TLC in TBA, 15% HOAc and visualized with NA spray. For standard loading of 2D-PCs, the extract from 90 mg of dry plant material was applied and the chromatograms were run in (i) *t*-BuOH-HOAc-H_2O (3:1:1) and (ii) 15% HOAc.

Biflavonoid mass spectra. The following MH^+ ions were detected as indicated. Agathisflavone 7-*O*-methyl ether (NBA/MeOH LSIMS 20 keV Cs^+) 553 amu; cupressuflavone 7,7″-dimethyl ether (NBA Matrix LSIMS 23 keV Cs^+) 567 amu; agathisflavone 7,7″-dimethyl (Glycerol LSIMS 23 keV Cs^+) 566 amu; cupressuflavone 7-*O*-methyl ether (NBA/MeOH LSIMS 20 keV Cs^+) 553 amu; agathisflavone 7,7″-dimethyl ether (NBA/CH_2Cl_2 LSIMS 20 keV Cs^+) 567 amu; cupressuflavone 4′,4‴,7,7″-tetramethyl ether (NBA/CH_2Cl_2 LSIMS 20 keV Cs^+) 595 amu.

Sugar and aglycone analyses. Standard hydrolysis methods, followed by co-chromatography against authentic standards [12]. Solvent and spray for sugar PCs: *n*-BuOH-pyridine-H_2O, 15:10:7.5, 3% aniline phthalate in MeOH.

Absorption spectra (λ_{max} nm). Cupressuflavone 7,7″-dimethyl ether: (MeOH) 274, 332, 360sh; (NaOMe) 272, 390; ($AlCl_3$) 280, 350, 392sh; ($AlCl_3$/HCl) 280, 346, 292sh; (NaOAc) 274, 348, 395; (NaOAc/H_3BO_3) 274, 338. Agathisflavone 7,7″-dimethyl ether: (MeOH) 274, 324; (NaOMe) 282, 310sh, 364sh, 390; ($AlCl_3$) 280, 305sh, 335; ($AlCl_3$/HCl) 274, 344; (NaOAc) 278, 388; (NaOAc/H_3BO_3) 274, 328. Cupressuflavone 7-methyl ether: (MeOH) 275, 332; (NaOMe) 285, 395; ($AlCl_3$ and $AlCl_3$/HCl) 275, 284sh, 306, 349, 395sh; (NaOAc) 283, 344, 395; (NaOAc/H_3BO_3) 275, 332. Cupressuflavone 4′,4‴,7,7″-tetramethyl ether: (MeOH) 274, 326, 360sh; (NaOMe) 274, 290, 305sh, 390sh.

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