Studies on the Chemistry of Lichens, XIX. New Amino Compounds from *Anaptychia fusca* and Several *Stereocaulon* Species

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The lichen *Anaptychia fusca* and several *Stereocaulon* species have been chemically investigated with regard to their content of unknown ninhydrin-positive compounds. New aliphatic and alicyclic, amino components isolated from water extracts of these lichen species have been characterised as aminopentitol, aminohexitol and aminocyclitetrol. To our knowledge, this is the first time that compounds such as these have been found in lichen material. Aminopentitol and aminocyclitetrol appear to be characteristic constituents of *Anaptychia fusca*, whereas aminohexitol has been found in several species of Stereocaulaceae.

Introduction

Owing to the discovery in recent years of a range of unusual aminosugars, frequently occurring as components of microbial metabolites, there is much current interest in the chemistry of this class of compounds. The isolation of a new compound from the lichen species *Xanthoria parietina,* established as *3-hydroxy-4,4-amino(hydroxymethyl)tetrahydrofurfuryl alcohol,* $C_6H_{13}NO_4$, has recently been reported from our institute [1]. However, this constituent may also be considered as *5-amino-1,5-dideoxy-glycopyranose.*

In a more comprehensive investigation of the free sugars and ninhydrin-positive compounds of several Norwegian lichen species [2], two unusual substances were observed in the species *Anaptychia fusca* and *Stereocaulon pascale.* Both compounds, which afforded typical colours after reaction in buffer solution with ninhydrin, were described by column chromatographic methods, but not identified. The purpose of the present paper is to describe the isolation and identification of these compounds, also to offer evidence in favour of their proposed formulation as *aminopentitol* and *aminocyclitetrol* (a mixture designated as Anaptychia-X) from *Anaptychia fusca* and *aminohexitol* (as Stereocaulon-X) from *Stereocaulon* species.

The isolated substances belong to the class of acyclic and alicyclic amino polyols. Cyclitols bearing amino or substituted amino groups in place of hydroxyl groups, the so-called inosamines, have been revealed as constituents of several antibiotics. As yet no inosamines have been found in nature in their free state and the most frequently encountered natural inosamines are bonded in antibiotics.

The isolated aminocyclitetrol (dideoxyinosamine) from *Anaptychia fusca* in this investigation has a configuration unprecedented in natural inositols. It is not rare to find more than one polyol in the same species and several cases exist where polyols of different configuration co-exist, even in the same species. The discovery of the new amino compounds in this investigation should stimulate much work concerning the still unknown compoments 2, 5, 6 and 12 as described in our report of 1970 on other lichen species [2].

The identification of the compounds is based on elementary analyses, IR, NMR and mass spectrometry. The new amino compounds have not yet been synthesised and their structure is not, therefore, fully established.

Experimental

Materials and isolation of the amino compounds. The main part of the species *Anaptychia fusca* (Huds.) Vain. (in all 5.54 kg) used in this investigation was collected on the island of Runde in July-August, 1968 – 76. Species of *Stereocaulaceae* (in all 9.31 kg) were collected mainly in Folldal during 1975 – 77. The lichen materials were cleansed of all extraneous plant materials, air-dried and pulverised. The materials were at first treated with chloroform, acetone and ethanol, and thereafter extracted using the solvent mixture of water-isopropanol-0.05 N HCl

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(8 + 1 + 1) by continously shaking for 20 h. The water extract was centrifuged, filtered and evaporated to a smaller volume. Absolute ethanol was then applied to the cloudy solutions increasing the volume by 3–4 times in order to remove polysaccharides and proteins, treated with activated charcoal (Darco G 60) and filter aid, and the filtrates once more reduced to small volumes. The pH of the concentrated extracts, which by this time were quite clear and colourless, were regulated to approx. 7–8 and passed through columns (290 × 65 cm) of cation exchange resin (Dowex 50 W, X8, 50–100 mesh, H^+). After washing with water the amino acids and other amino compounds were displaced from the columns with 2 N ammonia. The ammonia eluates were evaporated to dryness and traces of NH_3 removed in vacuum. The residues were then taken up in a solution of ammoniumacetate (0.0875 N, pH 8.3) and treated with the Amberlite exchange resin IRC 50, 22–45 mesh, in the NH_4^+ form. After removal of acidic and neutral amino acids by eluting with the ammoniumacetate solution, the basic unknowns Anaptychia-X and Stereocaulon-X, together with small amounts of lysine, histidine and arginine, were collected in the ammonia eluate (0.5 N). The ammoniacal solutions were evaporated to syrups which were redissolved in 50 ml sodium citrate buffer (0.2 M, pH 2.2) and finally filtered through a Millipore Filter (0.45 μm).

To obtain a sufficient quantity of Anaptychia-X and Stereocaulon-X from the purified citrate solutions for detailed examinations, orders of separations, 1490 and 690 respectively, were performed by ion-exchange chromatography on columns (190 × 9 mm I.D.) equipped with type Durrum high efficiency cation exchange resin (Pierce DC-6A). The column effluent was pumped at a rate of 40 ml/h and the column temperatures used were 50 and 40 °C for the isolation of Anaptychia-X and Stereocaulon-X respectively. The composition of the trisodium citrate buffers used was 0.35 M with respect to Na^+ (pH 5.28) and 0.38 M (pH 4.25) for the separation of the above fractions respectively. Aliquots (40 μl) of the extracts were transferred to the columns and washed in with a pH 2.2 citrate buffer (0.2 M Na^+). Fractions (11 ml) were collected automatically by means of an amino acid analyser and an aliquot (0.5 ml) of every fourth fraction was analysed for Anaptychia-X and Stereocaulon-X with the ninhydrine reagent. The eluates were later on applied to freshly regenerated columns of Dowex 50 W, X8, 50–100 mesh, H^+, in order to remove the sodium citrate. Anaptychia-X crystallised by storage in a desiccator over P_2O_5 and solid NaOH.

Analytical methods. Acetylation of Anaptychia-X and Stereocaulon-X were performed using acetic acid anhydride in dry pyridine (1 + 3) and refluxing for 30 min. The periodate oxidation of Anaptychia-X was carried out in darkness at 4 °C in a 0.2 M $NaHCO_3$ solution of 0.17 M $NaJO_4$ for 23 h. In these conditions the malondialdehyde obtained did not oxidise further in spite of the active methylene group hydrogens. The aldehyde was isolated as 2,4-dinitrophenylhydrazone.

Glucos- and mannosaminitol were prepared in our laboratory from the corresponding aminosugars by $NaBH_4$-reduction, and 1-amino-1-deoxyarabinitol and aminopentahydroxy cyclohexane (aminocyclipentol) using the methods of Winestock and Plaut [3] and Grosheintz and Fischer [4] respectively. Other standards were purchased from the commercial sources and were of guaranteed grade purity.

Using TLC technique on cellulose plates (MN 300) with the elution agent ethanol/amyl alcohol/ammonia/water (62 + 15 + 15 + 8), the presence of two components was observed in the fraction Anaptychia-X, both of which afforded typical colours with ninhydrine and dimethylaminocinnamic aldehyde. The R_f-values of the consisting two components of Anaptychia-X were 0.38 and 0.71; fructosamine (ref. stand.) 0.58.

The elemental analyses were carried out by Analytische Laboratorien, Elbach-BRD. The high resolution mass spectra (HrMS) has been obtained from the Technical University of Trondheim and Shrader Analytical Laboratories, Detroit-USA. Trimethylsilyl (TMS) derivatives were prepared rapidly with trimethylchlorosilane and analysed by HrMS. Proton nuclear resonance (NMR) spectra in DMSO-d_6 at frequency of 60 Mc.p.s. Peak positions are given in δ-values (ppm) referenced internally downfield from tetramethylsilane. Infrared (IR) spectra were obtained from KBr discs.

All concentration steps were carried out at re duced pressure (water aspirator) using a rotary evaporator operating at temperatures not exceeding 35 °C.

Acknowledgements

The major of the *Stereocaulaceae* species investigated in this work has been collected and identified

by cand. real. Tor Tønsberg, The University of Trondheim, College of Arts and Science, and for this our sincere thanks are due. I am deeply indebted to my wife and sons for all their substantial help in the collection of *Anaptychia fusca* and *Stereocaulon* species.

Results and Discussion

The purity of the isolated fractions Anaptychia-X and Stereocaulon-X used for the present investigations was confirmed by column chromatography on an amino acid analyser and by TLC. The position occupied by the elution peak corresponded to that determined previously [2], and only a single, symmetrical peak could be observed on the column chromatograms. Analyses by use of different column, buffer and temperature conditions on the amino acid analyser and by comparison with reference compounds, displayed that the fractions Anaptychia-X and Stereocaulon-X were neither identical with any of the following compounds; glucos-, mannos-, galactos-, fructos- or allosamine, glucos- and mannosaminitol; nor with aminopentahydroxy cyclohexane. The mentioned compounds all exhibited short retention times but were not identical to those of the isolated compounds. Anaptychia-X emerged from the column with a retention time close to that of arabinosaminitol, whereas the less basic compound Stereocaulon-X left the column almost at the same moment as mannosaminitol.

The ratio of the areas under the 440 nm and 570 nm absorption peak tracing for 11 standard amino acids, amino sugars and aminoalditols, was average found to 0.14, for Stereocaulon-X 0.14 and for Anaptychia-X 0.29. On the basis of the work published by Conkerton *et al.* [5] the high ratio value found for Anaptychia-X confirms the TLC analysis that this fraction actually is a mixture of two ninhydrin-positive components, henceforth designated as Anaptychia-X1 and X2. On the same reasoning it is assumed that Stereocaulon-X is a single component, a fact which was later confirmed.

Since the nitrogen atom is basic in the compounds themselves and reacts with ninhydrine, it must in all three cases be present as primary amino groups.

One of the most significant features is that the compounds Anaptychia-X1 and X2 isolated from *Anaptychia fusca* have not so far been found in extracts from 45 other lichen species examined in the years 1970–79 at our institute. This supports our remark in 1970 [2] that Anaptychia-X are specific components of the mentioned species. Xantholamine found in *Xanthoria parietina* [1] has also remained undetected in other species. The compound Stereocaulon-X has only been detected in the family Stereocaulaceae, and hitherto in the species *S. alpinum* Laur., *S. botryosum* Ach. em. Frey, *S. coniophyllum* Lamb, *S. condensatum* Hoffm., *S. dactylophyllum* Flörke, *S. delisei* Bory ex Duby, *S. evolutum* Graewe, *S. glareosum* (Sav.) Magn., *S. grande* (Magn.) Degel., *S. nanodes* Tuck., *S. pascale* (L.) Fr., *S. pileatum* Ach., *S. rivulorum* Magn., *S. saxatile* Magn., *S. spathuliferum* Vain., *S. symphycheilum* Lamb and *S. tomentosum* Fr. The species *S. articum* Lynge, *S. vesuvianum* Pers. and *S. groenlandicum* (Dahl) Lamb have also been investigated at our institute, but the presence of this component was rather uncertain.

Anaptychia-X

It was imposible to separate the two components X1 and X2 in the mixture isolated from *Anaptychia fusca.* However, it was expedient to carry out investigations on the mixture and this sufficed to unravel the structure of the components. The fraction Anaptychia-X is readily soluble in water and gives evolution of nitrogen when treated with nitrous acid. Unsaturation was not observed in the IR spectra, nor was hydrogen absorbed by hydrogenation. Neither elementary analyses, NMR, or IR indicated the presence of CH_3-groups bonded to carbon, nitrogen oxygen atoms.

The IR spectrum of Anaptychia-X showed alkyl group bands at 2900 and 1450 cm^{-1}, broad 3340 cm^{-1} band due to OH and NH stretchings, 1575 cm^{-1} (amide band), 1060 cm^{-1} (alcohol band), and 815 cm^{-1} due to out of plane band for NH group.

Chemical ionisation (isobutane) mass spectrum exhibited two important peaks at *m/e* 152 (R.I. 100%) and *m/e* 164 (R.I. 63%) corresponding to the molecular ions of the two components Anaptychia-X1 and X2 respectively. Metastable ions are measured at *m/e* 130.0, 118.0 and 98.5 associated with the transitions *m/e* 164→*m/e* 146, *m/e* 152→*m/e* 134, and *m/e* 132→*m/e* 114 respectively. In the E.I.MS the most abundant ions were seen at *m/e* 145 ($C_6H_{11}NO_3$), 133 ($C_5H_{11}NO_3$), 132 ($C_5H_{10}NO_3$, 100%), 120 ($C_4H_{10}NO_3$), 114 ($C_5H_8NO_2$), 102 ($C_4H_8NO_2$), 90

($C_3H_8NO_2$), 73 (C_3H_7NO or $C_3H_5O_2$), 72 (C_3H_6NO) and 60 (C_2H_6NO, 100%), fragments which are quite common in MS of polyols. On the basis of HrMS the elementary composition of the molecular ions was found to be $C_5H_{13}NO_4$ (*m/e* 151) and $C_6H_{13}NO_4$ (*m/e* 163), and the other ions at *m/e* 132, 120, 102, 90, 73, 72 and 60 could easily be explained as *m/e* 163 – CH_2OH, *m/e* 151 – CH_2OH, *m/e* 151 – H_2O – CH_2OH or *m/e* 163 – $CH(OH)CH_2OH$, *m/e* 151 – $CH(OH)CH_2OH$, $HO{=}CH-CH-CH_2$ (O bridge, +), $H_2N-CH_2-CH-CH$ (O bridge, +), and $H_2N{=}CH-CH_2OH$ respectively.

The mixture of X1 and X2 was readily acetylated, forming the two oily pentaacetates $C_{15}H_{23}NO_9$ (*m/e* 361) and $C_{16}H_{23}NO_9$ (*m/e* 373), confirmed by C.I.- and E.I.LrMS and HrMS. Other prominent peaks in the high mass range of the spectra were observed at *m/e* 330 ($C_{14}H_{20}NO_8$), 313 ($C_{14}H_{19}NO_7$), 301 ($C_{13}H_{19}NO_7$), 288 ($C_{12}H_{18}NO_7$), 270 ($C_{12}H_{16}NO_6$), 254 ($C_{12}H_{16}NO_5$), 246 ($C_{10}H_{16}NO_6$) and 240 ($C_{11}H_{14}NO_5$) corresponding to processes involving losses of CH_3COOH, CH_3COO, CH_3CO and $CH_2{=}C{=}O$ or CH_2OCOCH_3 groups from the molecular ions. Two strong metastable ions measured at approx. *m/e* 263 and 252 in a C.I.MS are associated with the transitions *m/e* 374→*m/e* 314 and *m/e* 362→*m/e* 302 respectively. This supports the fact that the acetylated Anaptychia-X consists of a mixture of the two pentaacetates $C_{15}H_{23}NO_9$ and $C_{16}H_{23}NO_9$, and that the last one must be a cyclic, saturated compound. The fragmentation *m/e* 330→*m/e* 240 in an E.I.MS is accompanied by the metastable ion mesured at approx. *m/e* 192. Two strong fragments in a HrMS was observed at *m/e* 102 and 72 corresponding to $H_2N-CH_2-CH{=}\overset{+}{O}-COCH_3$ and $CH_2{=}\overset{+}{N}H-COCH_3$. The base fragment of the pentaacetates is found at *m/e* 84 corresponding to C_4H_6NO with the molecular structure $H_2\overset{+}{N}{=}C{=}CH-CH-CH_2$ (O bridge).

IR of the acetyl-mixture exhibited υ_{max} at 1740 cm^{-1} (O-acetyl) and 1660 cm^{-1} (N-acetyl).

The trimethylsilyl ether derivatives of Anaptychia-X1 (MW 511, $C_{20}H_{53}NO_4Si_5$) and Anaptychia-X2 (MW 523, $C_{21}H_{53}NO_4Si_5$) exhibited no molecular peaks in the mass spectrum. Two important peaks in the high mass range of a HrMS, however, were found at *m/e* 348 (93%) $C_{14}H_{34}NO_3Si_3$ and *m/e* 336 (7%) $C_{13}H_{34}NO_3Si_3$ corresponding to the fragments (*m/e* 523 – $CHOSiMe_3$ – $SiMe_3$) and (*m/e* 511 – $CHOSiMe_3$ – $SiMe_3$).

The above evidence, combined with the elemental analyses of the acetyl-mixture (Table I), permitted the unequivocal conclusion that Anaptychia-X consists of the two components *aminopentitol* ($C_5H_{13}NO_4$) and *aminocyclitetrol* ($C_6H_{13}NO_4$).

Confirmatory evidence for the presence of an aminocyclitetrol in Anaptychia-X was adducted by the periodate oxidation to yield, among other things, *1,3-propane-dial* (malondialdehyde), which was isolated as its 2,4-dinitrophenylhydrazone. In a HrMS of the dihydrazone with molecular ion $C_{15}H_{12}N_8O_8$ at *m/e* 432 (16%), an important ion was seen at *m/e* 250 (3.4%) corresponding to the fragment $C_9H_8N_5O_4$, $[2,4\text{-}(NO_2)_2\text{-}Ph-NH{-}N{=}CH-CH_2-C{\equiv}\overset{+}{N}H]$. Furthermore, the formation of formaldehyde by the same oxidation showed that a primary alcohol group was present in the vicinity of a secondary alcohol group. The formation of malondialdehyde by the present periodate oxidation indeed may support the assertion that the methylene group in the C6 ring is in the neighbour-position to the carbon atom bearing the amino group, rather than a position between two hydroxyl groups. In the latter case the methylene group would probably be more active and the oxidation continued.

The NMR spectrum of the acetyl-mixture reveals four sharp signals as expected from the protons of the equatorial acetamido group (δ 1.78) and axial and equatorial acetoxy groups (δ 2.00, 2.05 and 2.10, 15 H) in the C6 ring [6], in addition to the O- and N-acetyl of the aminopentitol in the signal at δ 2.10. It is well established that axial acetyl groups absorb at higher δ-values than equatorial ones. The relative intensity of the signal at δ 1.78 harmonised well with

	% C	% H	% N	% O	% NH	MW
Observed	49.75	6.32	3.80	39.87	3.9	360
Calculated when equal amounts of $C_{15}H_{23}NO_9$ and $C_{16}H_{23}NO_9$	50.67	6.31	3.82	39.21	4.1	367

Table I. Elemental analysis of the acetylated mixture isolated from *Anaptychia fusca*. MW measured osmometric in chloroform and acetone.

the fact that it is an equal amount of the two components X1 and X2.

Stereocaulon-X

The fraction was isolated in syrupy condition, and series of analyses unambiguously demonstrated the presence of a single component. The IR spectrum shows the expected bands at 3300, 2930, 1595, 1490, 1400 and 1035 cm^{-1}, characteristic to compounds belonging to the aminoalditols. No bands could be seen between 1400 and 1350 cm^{-1}, and no O-, C-, or N-CH_3 groups were present.

Chemical ionisation mass spectrometry of Stereocaulon-X showed base peak at *m/e* 182 (M+1) corresponding to the component $C_6H_{15}NO_5$. Other abundant peaks in MS were observed at *m/e* 164, 150, 146, 132, 110, 102, 90, 73, 72, 69 and 60. An identical fragmentation pattern was obtained by C.I. and E.I.MS of glucosaminitol, and HrMS results gave elementary composition of the ions identical with those given for Anaptychia-X previously shown. Metastable ions at *m/e* 148.0, 116.2 and 86.6 are associated with the transitions *m/e* 182→*m/e* 164, *m/e* 150→*m/e* 132, and *m/e* 150→*m/e* 114 or *m/e* 120→*m/e* 102 respectively.

By acetylation the Stereocaulon-X readily formed the hexaacetate with the base peaks at *m/e* 433 (M^+, $C_{18}H_{27}NO_{11}$) and *m/e* 373 ($M^+ - CH_3COOH$) in a C.I.MS. Other prominent peaks were seen at *m/e* 391, 331 and 313 responsible to the fragments M-42, M-42-60 and M-2 · 60 respectively. The acetate exhibited absorption frequencies at the expectant positions 1730, 1665, 1630, 1480, 1420, 1365, 1220 and 1025 cm^{-1}.

Fully trimethylsilylated ether of Stereocaulon-X ($C_{24}H_{63}NO_5Si_6$, MW 613) does not demonstrate any molecular ion in HrMS. The most important primary fragmentation is shown to be cleavage of the carbon chain by C1-C2 and subsequent loss of two mol TMS, giving the fragment $C_{14}H_{36}NO_4Si_3$ at *m/e* 366 (1.4%). The formation of the *m/e* 348 (1.7%) $C_{14}H_{34}NO_3Si_3$ step occurs through loss of water. The most characteristic features of the remaining peaks is the high intensity ions at *m/e* 145 (25%) $C_6H_{13}O_2Si$ and *m/e* 132 (83%) $C_5H_{14}NOSi$ for the fragments $CH-CH-CH_2-O-TMS$ (with $\overset{+}{O}$) and $HO=CH-CH_2-\overset{+}{N}H-TMS$ respectively.

The prominent fragment obtained at *m/e* 307 ($C_{12}H_{31}O_3Si_3$), is formed by cleavage of the C3 to C4 bond and represents the fragment $TMS-\overset{+}{O}=CH-CH(O-TMS)-CH_2-O-TMS$. It apparently fragments further by eliminating trimethylsilanol and forming the commonly encountered *m/e* 217 ($C_9H_{21}O_2Si_2$) ion represented by the formular structure $CH_2=C(O-TMS)-CH=\overset{+}{O}-TMS$.

The analytical work leaves no doubt that Stereocaulon-X is an *aminohexitol,* $C_6H_{15}NO_5$, with the NH_2 group attached to carbon atom, C5 or C6.

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