ISOLATION AND STRUCTURAL DETERMINATION OF COMPOUND AM-4 FROM THE STEM BARK OF *Annona muricata* Linn

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**ABSTRACT**

*Annona muricata* Linn, belongs to the family Annonaceae. Oil from seeds of some plants may be used for the production of edible oils and soaps. Finally, many members of this family are used in folk medicines for various purposes. *Annona muricata* roots are used as anti-spasmodic, parasiticidal. Flower and flower buds as a bechic. The unripe fruits is useful as anti-scrobutic. Seeda are useful as fish-poison, insecticidal and astringent. The compounds that are isolated from *A. muricata* are Anomuricine, Anomurine, Coclaurine, Reticuline, Coreximine, Stepharine, Atherosperminine, Anonaine and Liriodenine. AM-4 was obtained as waxy solid, m.p 87-89°C, [α]D23 + 11.00 (c=0.4, MeOH), from the fraction slightly more polar than squamocin. AM-4, C37H66O8, (FAB-MS : m/z 639 (M+H)+), indicated the presence of α,β-unsaturated γ-lactone and hydroxyl groups. AM-4 was identified as Squamostatin-A on the basis of detailed spectroscopic analysis and comparison with authentic sample.  

**KEYWORDS:** *Annona muricata*, Squamostatin, Squamocin, 1H-NMR spectrum, Annonaceae.

**INTRODUCTION**

*Annona muricata* Linn, belongs to the family Annonaceae which is a large family of tropical and subtropical trees and shrubs comprising about 120 genera and more than 2000 species. Economically the family is of appreciable importance as the source of edible fruits, the pawpaw (*Asimina*), cherimoya, custard apples, sweet sop, sour sop and ilama (*Annona*) and fruits of the genera *Canaga* and *Rollinia*. Oil from seeds of some plants may be used for the production of edible oils and soaps. Finally, many members of this family are used in folk medicines for various purposes.
Morphological Descriptions: It is a small being glabrous when old. The leaves are large, ovate, obovate, acute or bluntly acuminate, rounded at the base, glabrous; blade 7-2.5 inch, thinly coriaceous, pellucid, punctate, lateral nerves about 12 pairs, prominently seen beneath; flowers in axillary; leaf opposed, pedicelled, few flowered racemes; sepals: triangular, shortly acuminate, pubescent; petals; greenish yellow, usually 3 in numbers, fleshy, triangular, united, thickened and saccate at the base, pubescent on both surfaces about 1 inch long. Pedicles stout; bracteates in the middle, thickened at the tip, one to 2 inch long; fruits: large, globouse, often of irregular growth; carpels do not separate (As in Asquamosa), each with an acute tip, giving the surface of the fruit a muricate appearance.

Geographical distribution: A. muricata is a native of West Indies. The tree occurs wild and is also cultivated in Cuba, St. bomingo, Jamaica, in gardens near Pune and Mumbai, in Assam and in South India. Apart from A. muricata, the following species of Annona are also reported to be available in India. A. squamosal, A. reticulate, A. glabra, A. cherimolia, A. perpurea, A. montana, A. senegalensis and A. atemoya.

Medicinal Uses: A. muricata finds a variety of medicinal uses in traditional system of medicine. The roots are used as anti-spasmodic, parasiticidal. Flower and flower buds as a bechic. The unripe fruits is useful as anti-scorbutic. Seeda are useful as fish-poison, insecticidal and astringent.

Chemistry of A. muricata: From the approximately 120 genera and more than 2000 species that are generally considered to make-up the Annonaceae, less than 50 genera and 200 species appear in the chemical literature at all. Even many of the phyto chemical studies of these family reported so far are at best fragmentary. Hance phyto chemical studies and to a lesser extent pharmacological studies on Annonaceous plants have been intensified in the last decade. Most investigations have centered upon allakaloids but Annonaceae also produce a wide range of compounds belonging to various phyto chemical groups. The review paper by Leboeuf et. al. covers the phyto chemistry of Annonaceae up to 1982 which include various allakaloids, carbohydrates, lipids, amino acids, proteins, poly phenols, essential oils, terpenes and aromatic compounds typically found in these plants. Apart from these components, different species of Annona have revealed the presence of a noval group of compounds named Annonaceous acetogenins. The compounds that are isolated from A. muricata are Anomuricine, Anomurine, Coclarine, Reticuline, Coreximine, Stepharine, Atherosperminine, Anonaine and Liriodenine.
PROCEDURE FOR ISOLATION OF CHEMICAL CONSTITUENTS FROM THE STEM BARK OF A. muricata

Milled stem bark of A. muricata (0.75 kg)

1. Extracted thoroughly with petroleum ether (60-80°C) for 16 hours in a soxhlet
2. Left at ambient temperature for 24 hours
3. Decantation

Viscous waxy solid
AM-A
1. Washed with petrol (50 ml×3)
2. Chromatographed over SiO₂
3. Eluted with EtOAc: MeOH::20:1

Supernatant

Viscous oil

Supernatant

Aqueous alcoholic solution
1. Concentrated to 0.31
2. Partitioned with MeOH:H₂O (9:1, 0.31)

Petrol soluble (discarded)

Supernatant

AM – 1
Yeild: 0.025 g

Preparing TLC with
EtOAc: MeOH:CHCl₃::10:1:4

Aqueous alcoholic solution
1. Washed with petroleum ether (100 ml×3)
2. Extracted with chloroform (200 ml×3)

Chloroform Extract
1. Washed and dried
2. Evaporated

Viscous Oil
AM-B
Yeild: 2.21 gm

Aqueous alcoholic solution
(discarded)

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Supernatant

AM – 2
Yeild : 0.035 gm

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2. Evaporated

Viscous Oil
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Petrol soluble (discarded)

Supernatant

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Yeild : 0.045 gm

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Petrol soluble (discarded)

Supernatant

AM – 4
Yeild : 0.03 gm

Chloroform Extract
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2. Evaporated

Viscous Oil
AM-B
Yeild: 2.21 gm

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STRUCTURAL DETERMINATION OF COMPOUND AM-4 FROM THE STEM BARK OF A. muricata

Compound AM-4 was crystallised as white needles, m.p. 87-89°C (from Et0Ac), [α]D23 + 11.00 (c=0.4, MeOH). The results of elemental analysis has established the molecular formula of AM-4 as C37H66O8, which is corroborated with mass spectrometrically derived molecular weight 538 (FAB – MS spectrum of AM-4 displayed a quasi-molecular ion peak at m/z 639 [(M+H)+]. AM-4 responded positive towards Kedde’s reagent indicating the presence of a,b-unsaturated g-lactone ring in the molecule.

The UV absorption maximum (λmax 210 nm) and the IR absorption band (νmax 1750 cm⁻¹) has further supported the presence of a, b – unsaturated g-lactone ring in AM-4. The IR spectrum has also shown the presence of hydroxyl grouping (νmax 3590 and 3400 cm⁻¹) in the molecule of AM-4. A comparison of the IR and UV spectral data and the molecular formula of AM-4 with those of other known annonaceous acetogenins it appeared, at a glance, that AM-4 is a close relative of squamocin and probably having one additional hydroxyl group somewhere on the linear backbone of squamocin.

The ¹H-NMR spectrum of AM-4 was recorded on the 500 MHz instrument in CDC13 solution. The spectrum exhibited signals for a primary methyl group at δ 0.89, 3H, 3 (J = 6.9 Hz), a low – field methyl doublet at δ 1.41, 3H, d (J= 6.8 Hz) probably due to the methyl bound to the carbon bearing an oxygen function, a methylene group at δ2.26, 2H, tt (J= 7.7 and 1.4 Hz) probably due to allylic methylene bound to another methylene group, signals foreight oxymethine protons from δ 3.38 to 3.92 and an additional oxymethine proton at δ 4.99 as qq (J=6.8 and 1.4 Hz), probably the oxymethine of the g -lactone ring, and one olefinic proton signal at δ 6.98, brs, probably associated with a,b - unsaturated g-lactone ring. The Table given below shows the chemical shifts, their integral proton count, splitting pattern, coupling constant and probable assignment of different signals discernible the ¹H-NMR spectrum of AM-4.

Table: 500 MHz ¹H NMR data of AM-4 in CDC13

<table>
<thead>
<tr>
<th>Chemical shift (in δ -scale)</th>
<th>Integral proton count</th>
<th>Splitting pattern (coupling constant)</th>
<th>Probable Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.89</td>
<td>3H</td>
<td>triplet (J = 6.9 Hz)</td>
<td>- CH₂ – CH₂ – CH₃</td>
</tr>
<tr>
<td>1.41</td>
<td>3H</td>
<td>doublet (J = 6.8 Hz)</td>
<td>- CH – O</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH₃</td>
</tr>
</tbody>
</table>
A careful comparison of $^1$H-NMR spectral data of AM-4 with those of squamocin has clearly revealed the presence of α, β - unsaturated γ-lactone ring (signals at d 1.41, d; 2.26 tt; 4.99, qq; and 6.98, brs) and the terminal alkyl group (the unsymmetrical triplet at d 0.89).

A $^{13}$C-NMR spectrum of AM-4 is consistent with the $^1$H-NMR spectral data and showed the signals for all the 37 carbons (2x-CH$_3$, 23x-CH$_2$-, 1x>C=CH, 9x>CH-0- and 1x>C=O). A comparison of $^{13}$C-NMR spectrum of AM-4 with that of squamocin (Table 9) has revealed that the former bears an additional –CH-O carbon signal with concomitant loss of the signal for one CH$_2$ carbon as compared to squamocin. This information indicated that the eighth oxygen atom present in AM-4 is in the form of a secondary hydroxyl group. In order to secure some chemical information about the nature of oxymethine protons present AM-4 acetylation experiment was carried out.

**Acetylation of AM-4**

Acetylation of AM-4 with acetic anhydride and pyridine at room temperature as well as at elevated temperature, yielded a tetraacetyl derivative of AM-4, C$_{45}$H$_{74}$O$_{12}$ [FAB-MS : M+H]$^+$ at m/z 807]. The IR spectrum of AM-4 tetraacetate has shown a complete absence of hydroxyl absorption thus indicated that all the hydroxyl groups present in AM-4 are acylable.

The 500 MHz $^1$H-NMR spectrum of tetraacetyl AM-4 showed in addition to the expected signals originated from α, β -unsaturated γ-lactone ring and terminal methyl moieties, signals for oxymethine protons at d 3.88 (2H, m), and d 3.96 (2H, m) probably due to four oxymethine protons associated with two tetrahydrofuran rings and the signals in the region from d 4.80-4.91 (3H, m) and 5.00 (1H, m) are due to the carbinyl hydrogens associated with four acetate groupings, the methyl of acetates appeared at d 2.03, 2.05, 2.07 and 2.08 (3H, S each).
The overlapping of the carbinyl hydrogens between \( d \, 4.80-4.91 \) (3H, \( m \)) and \( d \, 5.00 \) (1H, \( m \)) associated with four acetate groups did not allow us to correlate the relative position of these carbinyl hydrogens. This prompted us to look for some chiral acylating agent which may solve the problem and we found that (R)-(+)-(trifluoromethyl)phenyl acetyl [(R)-(+)MTPA ester of AM-4 has successfully separated these carbinyl hydrogen to a great extent. Therefore, (R)-(+)MTPA ester of AM-4 was prepared.

**Tetra (R)-(+)MTPA ester of AM-4**

Tetra-(+)MTPA ester of AM-4 was prepared under usual condition. The 500 MHz \(^1\)H-NMR spectrum of tetra-(+)MTPA ester of AM-4 was recorded in CDCl\(_3\).

The \(^1\)H-NMR spectrum has clearly shown the presence of a, b-unsaturated g-lactone ring in tetra-(+)MTPA ester of AM-4 and a terminal methyl group. Moreover, a careful examination of \(^1\)H-NMR spectrum of tetra(+)MTPA ester of AM-4 has indicated that there are three types of etherial oxymethines with an integral ratio of 1:1:2 respectively at \( d \, 3.67 \) (q), 3.74 (quinted), and 3.89 (m) suggesting that the hydrogen giving quartet signal at \( d \, 3.67 \) (1H) and the multiplet at \( d \, 3.89 \) (2H) have in their vicinity probably only three hydrogens and the quintet nature of the hydrogen signal at \( d \, 3.74 \) (1H) suggested the presence of four hydrogens in its vicinity. The methine signals bound to (+)-OMTPA ester were seen at \( d \, 4.91 \) (2H, m), 5.01(1H, quintet), and 5.16(1H, q). Extensive decoupling experiments, as shown in Figure-28, were carried out in order to correlate the relationship of these oxymethine proton signals in the molecules.

The irradiation at \( d \, 5.01 \) has shown no change in the region from \( d \, 3.65 \) to 5.20 while some change was noticed in the methylene region, thus indicated the presence of \(-\text{CH}_2\text{-CH(OMTPA)-CH}_2\)-unit in the tetra-(+)MTPA ester where –OMTPA group is bound to a methine flanked between two methylenes. Irradiation at \( d \, 3.89 \) has simplified the multiplet at \( d \, 4.91 \) while the quartet at \( d \, 5.16 \) was changed into triplet a similar observation was noticed when reverse decoupling was carried out, i.e., the signal at \( d \, 3.89 \) was simplified when irradiation at \( d \, 5.16 \) and 4.91 were carried out. Thus suggesting the presence of \(-\text{CH}_2\text{-CH(OMTPA)-CH(OR)-CH}_2\)-unit in AM-4 tetra-MTPA ester. Irradiation at \( d \, 4.91 \) had changed the splitting pattern of the signal at \( d \, 3.89 \) and the quartet at \( d \, 3.67 \) was also changed to a triplet. Thus, suggesting the presence of altogether three units of type \(-\text{CH}_2\text{-CH(OMTPA)-CH(OR)-CH}_2\)- where the pairs of underlined hydrogens appeared at \( d \, 5.16 \) and 3.89, 4.91 and 3.89 and 4.91 and 3.67. Further, the shape of underlined hydrogen collapsed...
into a *triplet* \((J=5.5 \text{ Hz})\) upon irradiation of the partner which is further in support of five membered tetrahydrofuran ring in the molecule.

The *quintet* nature of the oxymethine at \(d 3.74\) suggested the presence of one \(-\text{CH}_2\text{-CH(OR)-CH}_2\)- unit in the molecule. The last oxymethine at \(d 4.98\) *quartet* of quartet has already been shown to be due to the \(\gamma\)-lactone ring, as has already been discussed earlier. The supporting evidence to this was further, obtained by decoupling of the methyl doublet at \(d 1.41\) which collapsed the *quartet* of *quartet* into a *quartet* \((J=1.4 \text{ Hz})\) only.

The \(^1\text{H}-^1\text{H}\) COSY spectrum of AM-4 tetra-\((+)-\text{MTPA}\) ester (Figure-29) is also consistent with the decoupling experiment and thus the accumulated 1D- and 2D-NMR evidence strongly support that the molecule of AM-4 possess ten methylene and few subunits.

Now the problem left behind is to unite the subunits together with ten methylene to arrive at the final structure of AM-4. In order to solve this problem chemical degradation of AM-4 was sought to carry out. The lead tetraacetate oxidation of AM-4 was carried out and the resultant mixture of reaction products obtained after work-up was subjected to GC-MS analysis. A detailed analysis of the EI-MS obtained from the chromatogram obtained at 1.7 min after the injection has clearly suggested the presence of 5-hydroxyundecanal in the mixture of reaction products other chromatograms were not relevant and could not be identified. A direct evidence for the presence of 5-hydroxyundecanal was established by a GC-MS comparison of the reaction product with synthetic sample of 5-hydroxyundecanal.

The EI-MS obtained from both the samples gave the highest ion peak at \(m/z\ 168\) (due to the loss of water molecule form the molecular ion). The isolation of 5-hydroxyundecanal by lead tetraacetate oxidation can only the rationalized from the unit.

The presence of 1,5-dihydroxy moiety has further been verified by a detailed analysis of \(^{13}\text{C}\)-NMR spectrum of AM-4 which showed a triplet at \(22.0\). This triplet for a methylene can only be possible if there is a 1,5-dihydroxy grouping in the molecule.

**RESULT AND DISCUSSION**

On the basis of the data discussed so far the following structure of AM-4 can be determined –
The above structure for AM-4 was further confirmed by the mass spectral data. The FAB-MS/MS of AM-4 which shows the peaks arising directly form the quasi-molecular ion peak at m/z 639 (M+H)^+. In addition to this the spectrum also exhibits a series of peaks at m/z 621, 603, 585, 567 and 531 originated due to the sequential loss of water molecules from hydroxyl groups and oxygen of the tetrahydrofuran rings. The other important fragments were at m/z 433 (originated from C_{23}-C_{24} fission-H_2O), 415 (433-H_2O), 363 (C_{19}-C_{20}fission-H_2O), 345 (363-H_2O), 293 (C_{15}-C_{16} fission) and 275 (293-H_2O). The FAB-MS/MS shows the daughter ions arising form the ions at m/z 345, 319 and 97, and the m/z 293 ion afforded important daughter ions at m/z 275, 265. Thus the FAB-MS/MS of AM-4 was very useful because it showed the peaks originated from the daughter ions obtained from the quasi-molecular ion peak at m/z 639(M+H)^+.

CONCLUSION
Systemic fractionation of the petroleum ether extract of the bark of A. muricata led to the isolation of 4 compounds which were previously levelled as AM-1, AM-2, AM-3 and AM-4. AM-4 belonged to non-adjacent bis – tetrahydrofuranic acetogenin. Compound AM-4 was identified as Squamostatin –A. AM-1, AM-2 and AM-4 are reported to occur for the first time in the stem bark of this plant species.

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