Echinulin, a novel cyclic dipeptide carrying a triphenylated indole moiety from an Anacardiaceae, a Cucurbitaceae and two Orchidaceae plants: detailed high resolution 2D-NMR and mass spectral studies

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A levorotatory compound, C\textsubscript{29}H\textsubscript{39}N\textsubscript{3}O\textsubscript{2} was isolated from the edible fruits of \textit{Rhus parviflora} (Anacardiaceae) along with citric acid 2-methyl ester. A detailed \textsuperscript{1}H and \textsuperscript{13}C NMR spectral studies including \textsuperscript{1}H-\textsuperscript{1}H correlation, long range \textsuperscript{13}C-\textsuperscript{1}H correlation (HMBC and HMQC) and also NOESY confirmed its structure as C\textsubscript{29}H\textsubscript{39}N\textsubscript{3}O\textsubscript{2}. Its UV spectrum established the presence of an indole chromophore in the molecule, while its IR spectrum showed sharp absorption peaks for aromatic NH (3665), two amide NH groups (3290 and 3030) and two amide carbonyls (1677 and 1667 cm\textsuperscript{-1}). Its 300 MHz \textsuperscript{1}H NMR as well as 75 MHz \textsuperscript{13}C NMR spectra showed a large number of peaks which could be mostly assigned in structures 2 and 3, but necessitated further organization for correct informational pool. The unequivocal assignments of each carboxyl group.

\textit{Rhus parviflora} Roxb.\textsuperscript{1,2} (Fam. Anacardiaceae) (Nepali name: \textit{Satibayer}\textsuperscript{2}) is a large shrub or a small tree and grows in the western Himalaya from Kumaon to Nepal and in central India on Pachmarhi Hills. The edible fruits were collected from the eastern part of Nepal at an altitude of 1200 m (approx.). The fruits are yellowish red, 5–7 mm in diameter. They are used in Hindu medicine and when mixed with salt act like tamarind. The fruits are chewed by Nepalese when at work or travelling along the hill track to quench thirst and get energy. A citric acid derivative was isolated from these fruits in this laboratory which may be responsible for such relief. This compound\textsuperscript{3} identified as 2-hydroxy-1,2,3-propanetricarboxylic acid 2-methyl ester serves as an interesting example of a simple achiral molecule having three adjacent prochiral centres and two enantiotopic pairs of diastereotopic geminal hydrogens as demonstrated from its \textsuperscript{1}H NMR spectral data.

The chloroform extract of the dried fruits on silica gel chromatography yielded an amorphous material forming colourless crystals, m.p. 238–239\textdegree{} (from ethanol), $[\alpha]\textsubscript{D}$ \textsuperscript{3} = -36.3\textdegree{} (CHCl\textsubscript{3}), $M^+$ at $m/z$ 461 (C\textsubscript{29}H\textsubscript{39}N\textsubscript{3}O\textsubscript{2}). Its mass spectrum established the presence of an indole chromophore in the molecule, while its IR spectrum showed sharp absorption peaks for aromatic NH (3665), two amide NH groups (3190 and 3030) and two amide carbonyls (1677 and 1667 cm\textsuperscript{-1}).

Its 300 MHz \textsuperscript{1}H NMR as well as 75 MHz \textsuperscript{13}C NMR spectra showed a large number of peaks which could be mostly assigned in structures 2 and 3, but necessitated further organization for correct informational pool. The unequivocal assignments of all peaks and the unequivocal elucidation of its structure have been possible through extensive studies at much stronger magnetic fields of its \textsuperscript{1}H NMR (600 MHz) and \textsuperscript{13}C NMR (150 MHz) spectral studies including \textsuperscript{1}H-\textsuperscript{1}H correlation, homodecoupling, long range and one-bond, \textsuperscript{13}C-\textsuperscript{1}H correlation (HMBC and HMQC) and NOESY.

The \textsuperscript{1}H NMR spectrum (600 MHz) of echinulin (2) displayed discrete resonances accounting for all 39 protons present in the molecule. Information about the proton net-
works was secured primarily from $^{1}$H- $^{1}$H correlation spectral studies and some homodecoupling experiments. Final assignments of the $^{1}$H resonances as given in Table I and shown in structure 4, were made on the basis of NOESY experiment.

The $^{1}$H spectrum showed (structure 2) broad singlets (1H each) at $\delta$ 5.67 and 6.04 for NH protons of two CONH groups. Homodecoupling experiments established that these units were part of CONHCHCH$_{3}$ and CONH-CH-CH$_{3}$ moieties. Decoupling of $\delta$ 5.67 (br s) resonance simplified the multiplet resonance at $\delta$ 4.41 (H-9) while decoupling of the latter resonance affected $\delta$ 5.67 (br s) resonance, concomitant with the changeover of non-equivalent methylene proton (H$_{A}$-8 and H$_{B}$-8) resonances at $\delta$ 3.18 (dd) and 3.66 (dd) to doublets. Again, decoupling of $\delta$ 6.04 resonance simplified the multiplet resonance at $\delta$ 4.10 (H-12) while decoupling of the latter resonance simplified the former resonance and also changed the methyl resonance at $\delta$ 1.53 (d) to a singlet. The aforesaid observations were in conformity with the presence of -CONH-CH-CH$_{3}$ and -CONH-CH-CH$_{3}$ moieties in the molecule. Such conclusions were ably supported by mutual correlation observed in the homonuclear ($^{1}$H-$^{1}$H) correlation experiment.

The methylene proton signal at $\delta$ 3.39 (d, $J$ 7.1) showed mutual correlation with olefinic proton resonance at $\delta$ 5.35 (m) in the COSY spectrum. The latter signal also showed reciprocal correlation with the proton resonances at $\delta$ 1.736 (d, $J$ 1.0) and 1.74 (br s) for two olefinic methyl groups in consonance with the presence of a 3,3-dimethylallyl unit. Presence of another 3,3-dimethylallyl unit was ascertained from the observation of mutual $^{1}$H-$^{1}$H correlation between the methylene proton resonance at $\delta$ 3.53 and olefinic proton resonance at $\delta$ 5.42 (m) on one hand, while on the other hand between the said olefinic proton resonance at $\delta$ 5.42
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| Proton | δH  | 1H-1H correlation | NOESY correlation |
|--------|-----|-------------------|-------------------|
| H-1    | 8.05, br s | H-6, H-2", H-3", H-4, H-5" | H-2'" - H-3'" |
| H-4    | 7.13, br s | H-6 | H-2", H-3", H-4, H-5" |
| H-6    | 6.81, br s | | |
| H-8    | 3.18, dd, (14.8, 11.7) | H-8, H-9, H-3", H-4, H-5" | |
| H-9    | 3.66, dd, (14.8, 3.8) | H-8, H-9 | H-4, H-5" |
| H-11   | 4.41, m | | H-4, H-5", H-11-14 |
| H-12   | 4.10, s | | H-11, H-15 |
| H-14   | 5.67, br s | | H-19 |
| H-15   | 1.53, d, (7.1) | | H-12 |
| H-2'   | 6.10, dd, (17.3, 10.5) | H-3", H-4", H-3", H-4" | |
| H-3'   | 5.15, dd, (10.5, 1.0) | H-2", H-3", H-4" | H-2' |
| H-5'   | 5.16, dd, (17.3, 1.0) | H-2", H-3", H-4" | H-2' |
| H-6'   | 3.39, d, (7.1) | H-3", H-4, H-6", H-2", H-3" | H-4, H-6", H-2", H-3" |
| H-7    | 5.35, m | | |
| H-3"   | 1.736, d, (1.0) | H-2'" | H-2'"

The 13C NMR spectrum displayed (structure 3) resonances for seven methyl carbons (at δ 17.89, 17.9, 19.9, 25.7, 25.8, 27.8 and 27.9), four methylene carbons (at δ 29.4, 31.4, 34.6 and 112.3), seven methine carbons (at δ 50.8, 54.5, 115.0, 122.9, 122.9, 124.5 and 145.7), and eleven non-protonated carbons (at δ 39.0, 104.1, 123.4, 128.9, 131.6, 132.2, 132.9, 133.9, 141.4, 167.7 and 168.3). Resonance assignments (vide Table 2) for the protonated carbons were established by their inverse 1H-13C heteronuclear correlation (HMBC) experiment where a proton signal correlated with the resonance of the carbon to

| Carbon | δC  | HMQC correlation (1JCH) | Long range 13C-1H correlation (HMBC) |
|--------|-----|-------------------------|--------------------------------------|
| C-2    | 141.4, s | H-4 | H-4, H-5, H-4' |
| C-3    | 104.1, s | H-4 | H-4, H-5, H-4' |
| C-3a   | 128.9, s | H-8 | H-8 |
| C-4    | 115.0, d | H-12 | H-12, H-13 |
| C-5    | 133.9, s | H-11 | H-11, H-12 |
| C-6    | 122.9, d | H-13 | H-13, H-14 |
| C-7    | 123.4, s | | |
| C-7a   | 132.2, s | | |
| C-8    | 29.4, t | H-9 | H-9, H-10 |
| C-9    | 54.5, d | | |
| C-10   | 168.3, s | | |
| C-12   | 50.8, d | H-12 | H-12 |
| C-13   | 167.7, s | H-11 | H-11, H-12 |
| C-15   | 19.9, q | H-13 | H-13, H-14 |
| C-1'   | 39.0, s | H-14 | H-14, H-15 |
| C-2'   | 145.7, d | H-2' | H-2', H-3', H-4' |
| C-3'   | 112.3, t | H-3' | H-3', H-4' |
| C-1'' | 34.6, t | H-2' | H-2', H-3', H-4' |
| C-2'' | 124.5, d | H-2' | H-2', H-3', H-4' |
| C-3'' | 131.6, s | H-2' | H-2', H-3', H-4' |
| C-4'' | 25.7**, q | H-3', H-4', H-3', H-4' |
| C-5'' | 17.89**, q | H-3', H-4' | H-2', H-3', H-4' |
| C-6'' | 31.4, t | H-2' | H-6 |
| C-7'' | 122.9, d | H-2' | H-2', H-3', H-4' |
| C-8'' | 25.8**, q | H-3', H-4' | H-2', H-3', H-4' |
| C-9'' | 17.9, q | H-5' | H-2', H-3', H-4' |
| C-10''| 27.8, 27.9, q | H-3', H-4', H-3', H-4' |

(dd, H-5') and also with indole NH resonance at δ 8.05. Again, the aromatic proton signal at δ 7.13 showed NOESY signals with proton resonances at δ 3.18 (dd), 3.66 (dd) and 4.41 (m) in consonance with the linkage of the remaining part of the molecule to C-3 of indole unit. The results are summarized in structure 1.

The 13C NMR spectrum displayed (structure 3) resonances for seven methyl carbons (at δ 17.89, 17.9, 19.9, 25.7, 25.8, 27.8 and 27.9), four methylene carbons (at δ 29.4, 31.4, 34.6 and 112.3), seven methine carbons (at δ 50.8, 54.5, 115.0, 122.9, 122.9, 124.5 and 145.7), and eleven non-protonated carbons (at δ 39.0, 104.1, 123.4, 128.9, 131.6, 132.2, 132.9, 133.9, 141.4, 167.7 and 168.3). Resonance assignments (vide Table 2) for the protonated carbons were established by their inverse 1H-13C heteronuclear correlation (HMBC) experiment where a proton signal correlated with the resonance of the carbon to

(m) and methyl proton resonance at δ 1.81 (d, J 1.0) as well as at δ 1.87 (br s). The presence of a monosubstituted olefin unit was also discernible from the COSY spectrum. Further, the 1H NMR spectrum also displayed two methyl signals at δ 1.508 and 1.511 as singlets implying the presence of a 1,1-dimethylethyl part in the molecule.

The molecule also had two aromatic proton resonances at δ 6.81 (br s) and 7.13 (br s) showing mutual correlation among themselves in COSY spectrum and another broad singlet at δ 8.05 (br s) for indole NH proton. Their relative dispositions were ascertained from NOESY experiment.

The aliphatic methylene proton signal at δ 3.39 (d) showed NOESY correlation with both the aromatic proton resonances at δ 6.81 (br s) and 7.13 (br s) while the NOESY correlation peaks were discernible between the other aliphatic methylene proton resonance at δ 3.53 (d) and the aromatic proton resonance at δ 6.81 as well as the indole NH resonance at δ 8.05 (br s). These observations established the presence of the 3,3-dimethylethyl units at C-5 and C-7 of the indole unit. Methyl resonances at δ 1.508 (s) and 1.511 (s) displayed mutual NOESY correlation with both the olefinic proton signals at δ 6.10 (dd, H-2') and 5.15
which the said proton is attached. It was observed that the aliphatic methylene carbon resonances at δ 31.4 and 34.6 have one-bond correlation with the methylene proton resonances at δ 3.53 (br d) and 3.39 (br d), respectively, and they were thus for C-1’’’ and C-1’’, respectively. The other aliphatic methylene carbon resonance at δ 29.4 exhibiting correlation spots with resonances at δ 1.18 (dd) and 3.66 (dd) was thus for C-8. The olefinic methylene carbon signal at δ 112.3 (C-3’) correlated with geminal olefinic proton resonances at δ 5.15 (dd) and 5.16 (dd).

The mutual correlation of the aliphatic methine carbon resonances at δ 50.8 and 54.5 with the proton resonances at δ 4.10 (m) and 4.41 (m) identified them for C-12 and C-9, respectively. Further, it was noted that the sp² hybridized methine carbon resonances at δ 115.0 and 122.9 were for C-4 and C-6, respectively, while those at δ 122.9, 124.5 and 145.7 were for C-2’’’, C-2’’ and C-2’, respectively, as they had 1JC-H correlation with H-4, H-6, H-2’’’, H-2’’ and H-2’ resonances. Similarly, the resonance positions for various methyl carbons as summarized in Table 1 were identified. The resonance positions for non-protonated sp² carbons C-2, C-3, C-3a, C-5, C-7, C-7a, C-6, C-13, C-3’’ and C-3’’’ were conclusively ascertained from the analysis of HMBC spectrum of echinulin (I). It was observed that (i) C-2 signal (δ 141.4) displayed multiple bond correlation with H-A-8 and H-B-8 signals, (ii) C-3 resonance (δ 104.1) with H-1, H-A-8, H-B-8 and H-4 resonances, (iii) C-3α signal (δ 128.9) with H-B-8 signal, (iv) C-5 resonance (δ 133.9) with H-2-1’’’ signal, (v) C-7 signal (δ 123.4) with H-2-1’’’ signal, (vi) C-7α resonance (δ 132.2) with H-4, H-6 and H-2-1’’’ signals, (vii) C-10 signal (δ 168.3) with H-9 and H-14 resonances, (viii) C-13 resonance (δ 167.7) with H-11 and H-12 signals, (ix) C-3’’ signal (δ 131.6) with H₂-1’’, H₂-4’’, H₂-5’’’ resonances and (x) C-3’’’ signal (δ 132.9) with H₂-1’’, H₂-4’’ and H₂-5’’’ resonances. The results are summarized in structure 5.

**Mass fragmentation:**

The mass fragmentation pattern of echinulin is well consistent with its structure. The genesis of the major peaks is delineated in Scheme 1.

**Biogenesis:**

Echinulin^{4,5} (I) has been isolated earlier from the mycelium of the moulds of the Aspergillus echinulatus and Aspergillus glauca groups as an important constituent. It has been shown to be 2-(1,1-dimethylallyl)-5,7-bis(3,3-dimethylallyl)-3-(6-methyl-2,5-dioxopiperizinyl)methylindole on the basis of its degradative and spectroscopic studies as well as from some model experiments related to the synthesis of echinulin^{6}. This mould metabolite being bio-
genetically interesting as it has probable origin from tryptophan, alanine and three isoprene units, has evolved much interest of a number of research groups involved in the biosynthetic studies of natural products with special reference to indole alkaloids. This conjecture has been supported by the incorporation of \( \text{L-tryptophan} \) into echinulin in the mould *Aspergillus glauca* as well as *Aspergillus amstelodamiae*. The homochirality being the hallmark in living systems, from biogenetic point of view the cyclic peptides are formed from \( \text{L-amino acids} \) and this acceptance settles the stereochemistry of two asymmetric centres as \( S \). However, in the review by Grundon et al. the structure of echinulin has been drawn in such a way that the configuration of asymmetric centres appeared to be \( R \).

The echinulin has also been isolated from the chloroform extracts of both *Dendrobium fimbriatum* (Orchidaceae) and *Cimbidium aloifolium* (Orchidaceae) and also from the peels of the fruits of *Trichosanthes dioica* (Cucurbitaceae) widely used as vegetables.

Since the occurrence of echinulin was not known in higher plants prior to our report, initially we took it as a new cyclic peptide. However, literature survey settles its identity with a mould metabolite isolated from mycelium of the moulds of *Aspergillus glauca* groups.

**Experimental**

M.ps. measured in open capillary tubes are uncorrected.

IR spectra (KBr) were recorded on a Perkin-Elmer spectrophotometer. \( ^1H \) NMR (600 MHz) and \( ^{13}C \) NMR (150 MHz) spectra were recorded on a Varian-600 spectrometer in CDCl\(_3\) (unless mentioned otherwise); the TMS (\( \delta \) 0.00) or solvent (CHCl\(_3\) \( \delta \) 7.26 and CDCl\(_3\) \( \delta \) 77.0) was used as the internal standard and \( J \) values are expressed in Hz. The mass spectra were recorded at 70 eV. The silica gel 60–120 mesh (Qualigens) and silica gel 230–400 mesh (SRL) were used for column chromatography and flash chromatography. Petrol refers to b.p. 60–80\(^\circ\) fraction. The eluate fractions were monitored using microscopic slides with silica gel G layer put on them by dipping in its chloroform slurry, taking out and drying in ambient temperature; similar fractions were combined.

**Extraction**: Air-dried and powdered (450 g) fruits of *R. parviflora* was extracted exhaustively in a soxhlet apparatus with chloroform and methanol successively for 40 h each. The extracts were evaporated in vacuo to afford residues A (15 g) from chloroform and B (30 g) from methanol, respectively, which were chromatographed over silica gel (60–120 mesh) using solvents and solvent mixtures of increasing polarity. Fractions of a similar composition, as indicated by TLC, were combined.

**Isolation of echinulin** (1): The later petrol-ethyl acetate (3 : 2) eluate fractions of the main chromatogram of the chloroform extract afforded echinulin (1) as an amorphous white solid, crystallizing from ethanol as white needles (10 mg), m.p. 238–239\(^\circ\), \( [\alpha]_D \) = 36.3 (CHCl\(_3\)), M\(^+\) at \( m/z \) 461.

Air-dried orchids, *Dendrobium fimbriatum* and *Cimbidium aloifolium* as well as the dried peels of the fruits of *Trichosanthes dioica* (Cucurbitaceae) were separately extracted with petrol and chloroform respectively in a soxhlet apparatus. Each chloroform extract was chromatographed as above and yielded echinulin in 0.0015, 0.0010 and 0.0005%, yield, respectively.

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