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Application of azide–alkyne cycloaddition 'click chemistry' for the synthesis of Grb2 SH2 domain-binding macrocycles

Won Jun Choi,[a] Zhen-Dan Shi,[a] Karen M. Worthy,[b] Lakshman Bindu,[b] Rajeshri G. Karki,[a] Marc C. Nicklaus,[a] Robert J. Fisher[b] and Terrence R. Burke, Jr.[a,*]

[a]*Laboratory of Medicinal Chemistry, CCR, NCI, NIH, Frederick, MD 21702, USA*
[b]*Protein Chemistry Laboratory, SAIC-Frederick, Frederick, MD 21702, USA*

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Abstract—Copper (I) promoted [3+2] Huisgen cycloaddition of azides with terminal alkynes was used to prepare triazole-containing macrocycles based on the Grb2 SH2 domain-binding motif, 'Pmp-Ac_6c-Asn', where Pmp and Ac_6c stand for 4-phosphonomethylphenylalanine and 1-aminocyclohexanecarboxylic acid, respectively. When cycloaddition reactions were conducted at 1 mM substrate concentrations, cyclization of monomeric units occurred. At 2 mM substrate concentrations the predominant products were macrocyclic dimers. In Grb2 SH2 domain-binding assays the monomeric (*S*)-Pmp-containing macrocycle exhibited a K_d value of 0.23 μM, while the corresponding dimeric macrocycle was found to have greater than 50-fold higher affinity. The open-chain dimer was also found to have affinity equal to the dimeric macrocycle. This work represents the first application of 'click chemistry' to the synthesis of SH2 domain-binding inhibitors and indicates its potential utility.

The growth factor receptor-bound protein 2 (Grb2) is an SH2 domain-containing signal transducer that represents an attractive therapeutic target.[1] For Grb2 SH2 domains, where open-chain 'pTyr-Xxx-Asn' sequences are preferentially recognized in type-I β-turn conformations, induction of turn geometries through macrocyclization using ring-closing olefin metathesis[2] (RCM) has resulted in a number of potent binding inhibitors.[3] Recent reports have highlighted the growing utility in drug discovery of copper (I) promoted [3+2] Huisgen cycloadditions of azides with terminal alkynes, which is one example of 'click chemistry'.[4] This led us to examine the potential usefulness of this approach to prepare Grb2 SH2 domain-binding antagonists, including macrocyclic inhibitors. To date few reports of ring closure using azide–alkyne click chemistry have appeared for peptides or peptide mimetics.[5] Therefore, the current study was undertaken to examine click chemistry in the synthesis of Grb2 SH2 domain-binding inhibitors.

Macrocyclization using ring-closing [1,3] dipolar cycloaddition was undertaken using the protected monomeric sequences **8** that contain the tripeptide motif, 'Pmp-Ac_6c-Asn-amide' (Scheme 1). (Here Pmp represents the phosphatase-stable pTyr mimetic 4-phosphonomethylphenylalanine[6] and Ac_6c stands for 1-aminocyclohexanecarboxylic acid[7]). Preparation of **8** began with the known protected amine **1**, which was obtained by Mitsunobu coupling of phthalimide with commercially available 4-pentyn-1-ol.[8] Direct hydrazinolysis of **1** to yield 1-amino-4-pentyne had previously been reported to fail or to require tedious ion exchange chromatographic workup if an alternate reductive phthalimide cleavage procedure was used.[9] However, we were able to employ **1** as a source of 1-amino-4-pentyne by reacting this material with hydrazine in aqueous EtOH (reflux, 2 h),[10] then cooling the reaction mixture to room temperature, filtering, and concentrating the volatile product. Without further purification the crude amine was coupled directly with *N*-Boc L-Asn using active ester protocols [1-hydroxybenzotriazole (HOBT) and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC)]. This provided the N-Boc-Asn[1-(5-pentynyl)] amide **2** in 48% yield from **1**. Conversion of **2** to the *N*-Fmoc protected dipeptide **4** required initial TFA-mediated *N*-Boc deprotection followed by neutralization and chromatographic purification. Active ester

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* Corresponding author. Tel.: +1 301 846 5906; fax: +1 301 846 6033; e-mail: tburke@helix.nih.gov

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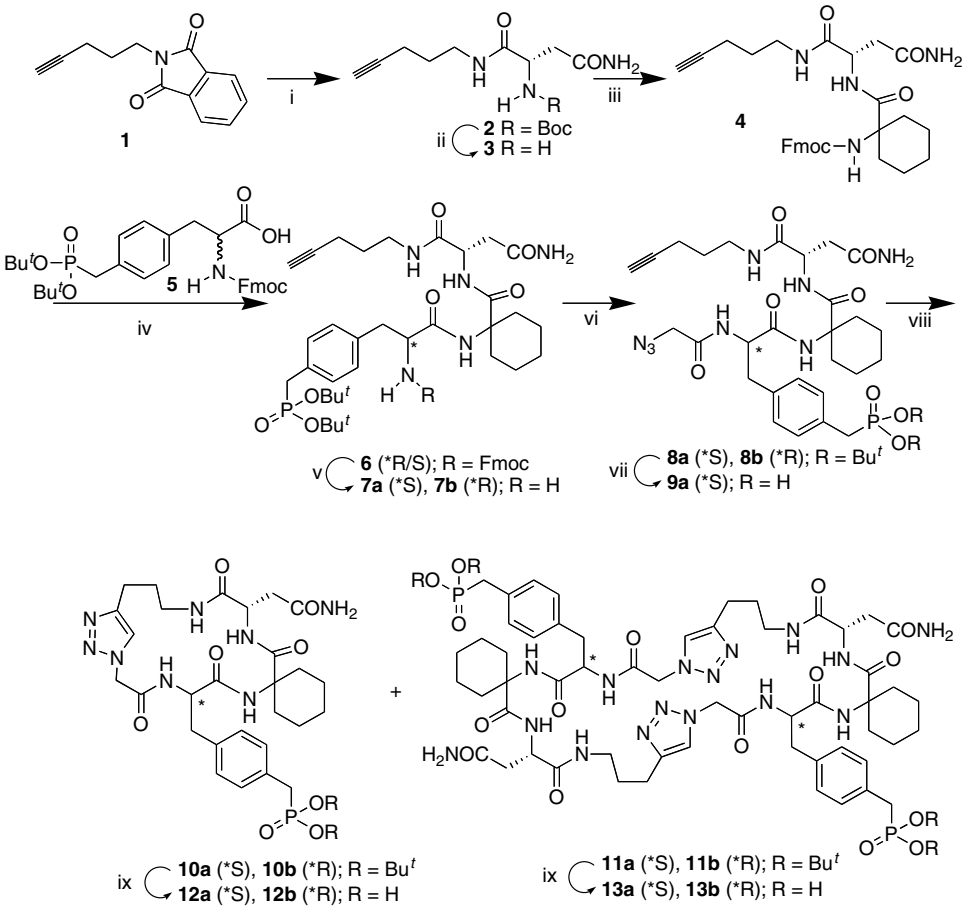
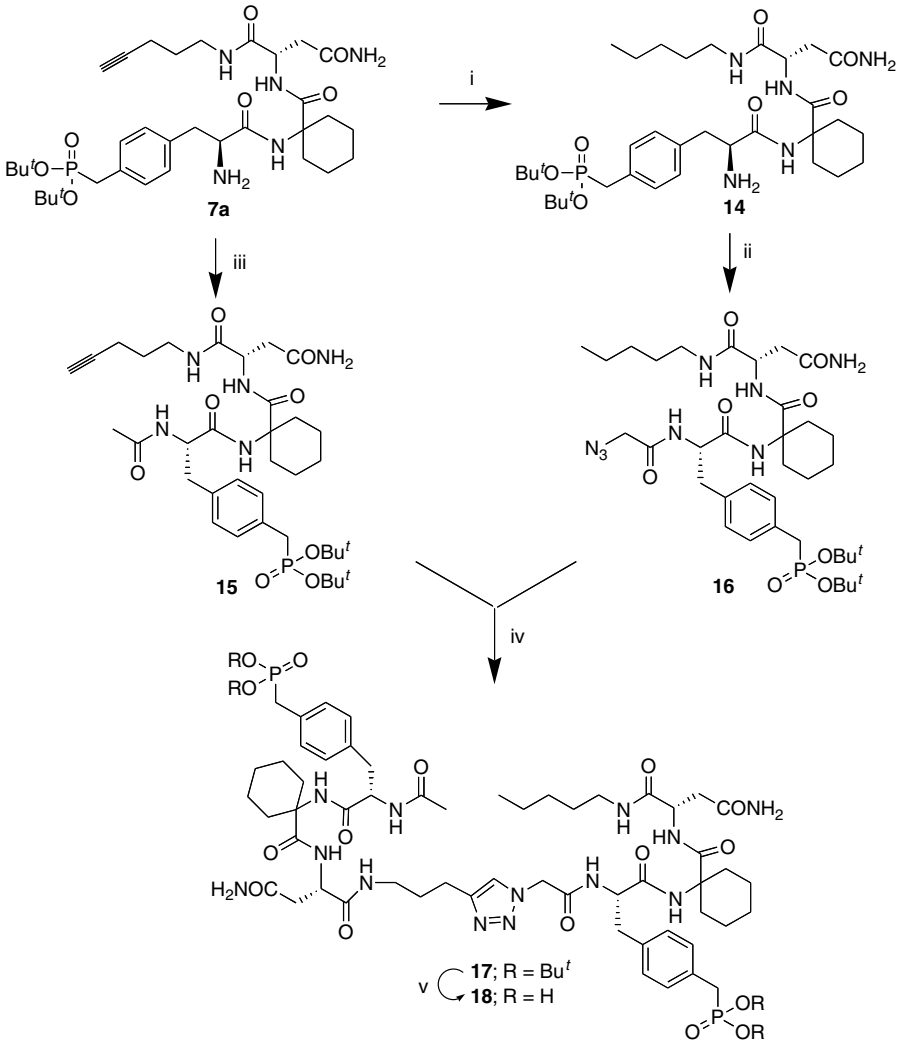


Scheme 1. Reagents and conditions: (i) a—$N_2H_4{\cdot}H_2O$, EtOH, H_2O, reflux, 2 h; b—Boc-Asn-OH, EDC, HOBt, DMF, rt, 12 h (48% yield for two steps); (ii) a—TFA, CH_2Cl_2, rt, 1 h (95% yield); (iii) a—Fmoc-1-amino-cyclohexanecarboxylic acid, EDC, HOBt, DMF, rt, 12 h (84% yield); (iv) a—piperidine, CH_3CN, rt, 2 h; b—EDCI·HCl, HOBt, DMF, rt, 12 h (39% yield for two steps); (v) piperidine, CH_3CN, rt, 2 h (37% yield for **7a**; 38% yield for **7b**); (vi) a—$(BrCH_2CO)_2O$ DMF, rt, 1 h; b—NaN_3, DMF, rt, 24 h (two steps, 81% yield for **8a**, 62% yield for **8b**); (vii) TFA–$HS(CH_2)_2SH$–H_2O, rt, 1 h (69% yield); (viii) CuI, L-ascorbate, DIPEA, CH_3CN/tBuOH/H_2O, rt; (ix) TFA–$HS(CH_2)_2SH$–H_2O, rt, 1 h.

coupling of the resulting free amine with *N*-Fmoc Ac_6c provided **4** in 80% yield from **2**. Removal of *N*-Fmoc protection followed by coupling with 4-[bis(*tert*-butyl)phosphonomethyl]-*N*-Fmoc-D,L-phenylalanine ([*N*-Fmoc-D,L-$(OBu^t)_2$Pmp], **5**)[11] gave the tripeptide **6** as an inseparable mixture of (*R*/*S*) epimers at the Pmp α-carbon. Following *N*-Fmoc removal, the epimeric free amines could be separated by flash chromatography to yield the (*S*)-Pmp- and (*R*)-Pmp-containing diastereomers **7a** and **7b**, respectively. Assignment of relative configurations was done by comparison with an authentic sample of (*S*)-Pmp-containing tripeptide (corresponding to the faster eluting material) prepared using enantiomerically pure *N*-Fmoc-L-$(OBu^t)_2$Pmp.[12] Treatment of **7a** and **7b** with bromoacetic anhydride, followed after 1 h by the addition of sodium azide, yielded the cycloaddition precursors **8a** and **8b** in 81% yield and 62% yield, respectively.

Initial attempts at [1,3] Huisgen cycloaddition using CuI and L-ascorbate[13] proceeded poorly and yielded mainly unreacted starting material. However, addition of *N*,*N*-diisopropylethylamine (DIEA) significantly facilitated the reaction, apparently by promoting formation of the copper-(I)-alkyne complex.[14] When the cycloaddition reaction was conducted using a substrate concentration of 2 mM, the expected monomeric cyclization product **10** was obtained only as a minor component. The major product under these conditions proved to be the dimeric macrocycle **11** (26% yield for **11a**, 56% yield for **11b**). The dimerization reaction was highly concentration dependent, since repeating the procedure at 1 mM substrate concentration reversed the product ratios and provided monomeric **10** (29% yield for **10a** and 19% yield for **10b**) as the predominant product with dimeric **11** appearing as a minor component. Similar peptide cyclodimerization by copper-catalyzed azide–alkyne cycloaddition has recently been reported.[5a] Cleavage of the phosphonate *tert*-butyl esters (TFA–ethanedithiol–H_2O) and purification by HPLC gave the final monomeric (**12**) and dimeric (**13**) products, respectively.

For comparison purposes the (*S*)-Pmp-containing open-chain monomer (**9a**) (Scheme 1) and dimer (**18**) (Scheme 2) were prepared. In the latter case, starting from the (*S*)-Pmp-containing tripeptide **7a**, cycloaddition precursors **15** and **16** were synthesized bearing C-terminal alkyne and N-terminal azide functionality, respectively. Performing [3+2] cycloaddition of **15** and **16** using CuI



Scheme 2. Reagents and conditions: (i) Pd/C, H_2, EtOAc; (ii) a—$(BrCH_2CO)_2O$ DMF, rt, 1 h; b—NaN_3, DMF, rt, 24 h (93% yield from **7a**); (iii) 1-acetylimidazole, DMF, 24 h (quantitative); (iv) CuI, L-ascorbate, DIPEA, CH_3CN/tBuOH/H_2O, rt (73% yield); (v) TFA–$HS(CH_2)_2SH$–H_2O, rt, 1 h (76% yield).

and L-ascorbate in the presence of DIEA gave the protected product **17**, which was converted to **18**.

Evaluation of Grb2 SH2 domain-binding affinities was conducted using surface plasmon resonance (SPR) as previously described.[15] A key feature of this technique was its use of Biacore S51 instrumentation, which allowed direct measurement of synthetic peptide binding to surface-bound Grb2 SH2 domain protein. In similar studies conducted on the reference macrocycle **19** (Fig. 1) using a Biacore 3000 instrument, close agreement was observed between the SPR binding affinity (K_d = 0.9 nM) and affinity determined by ELISA methods (IC_{50} = 1.4 nM).[3d]

As shown in Table 1, the (*R*)-Pmp-containing monomeric and dimeric macrocycles (**12b** and **13b**, respectively) exhibited binding constants above 1 μM. However, the monomeric (*S*)-Pmp-containing macrocycle (**12a**)[16] bound with sub-micromolar affinity. Of particular note were the affinities of the dimeric (*S*)-Pmp-containing peptides, which provided low nanomolar K_d values for

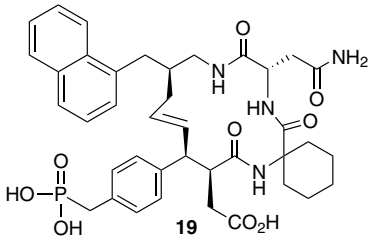


Figure 1. Structure of reference macrocycle examined by both SPR and ELISA binding experiments.

Table 1. Grb2 SH2 domain-binding affinities of synthetic peptides

Compound	Binding affinity K_d[a] (μM)
9a	>1
12a	0.23
12b	>1
13a	K_{d1} = 0.0018; K_{d2} = 0.0040
13b	>1
18	K_{d1} = 0.0011; K_{d2} = 0.087

[a] Values were determined as described in Ref. 15.

both the macrocyclic (**13a**)[17] and open-chain (**18**)[18] forms. These high affinities were somewhat unexpected based on the low affinity of the open-chain precursor **9a** ($K_d > 1$ μM).

The binding kinetics of **13a** and **18** were complex and consistent with multiple binding components. The quality of the Biacore data was very high and this was not felt to be an artifact of the data. Multiple binding components have been reported previously for Grb2 SH2 domain-binding macrocycles, however the physical interpretation is not clear.[19] Fitting data to a two-component system (Fig. 2) provided the K_{d1} and K_{d2} values shown in Table 1.

High Grb2 SH2 domain-binding affinity has been reported previously for a peptide containing two pTyr mimetic residues.[20] However, in this latter case both residues were situated in the proximal pTyr and pTyr+1 positions with the pTyr+1 residue participating in a non-canonical interaction with the Grb2 Arg142.[21] Molecular modeling indicates that this type of interaction is not possible for dimeric peptides **13a** and **18** (Fig. 3).

Peptides based on immunoreceptor tyrosine activation motifs (ITAMs) also present tandem pTyr-containing motifs that bind with high affinity to targets such as the ZAP-70 tyrosine kinase that contain multiple SH2 domains. X-ray analysis has confirmed that this binding involves simultaneous interaction of each pTyr-containing motif with a separate SH2 domain.[22] To our knowledge, peptides **13a** and **18** are among the first examples of dimeric pTyr-mimetic-containing peptides that show high affinity against a single SH2 domain construct. The structural basis for the more than two orders of magnitude affinity enhancement in going from the monomer **9a** to the dimers **13a** and **18** is not apparent from docking studies with the isolated Grb2 SH2 domain.

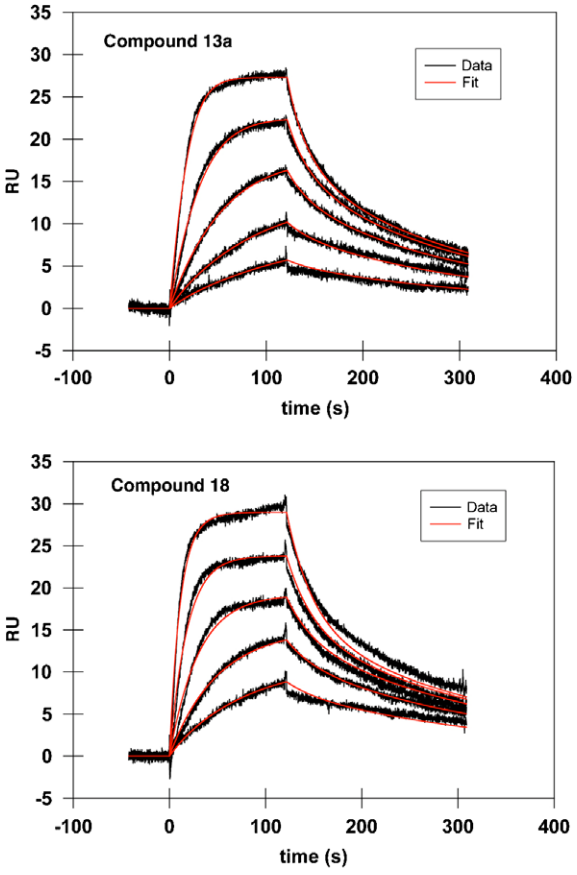


Figure 2. Representative SPR data for **13a** and **18** showing response in RU units versus time. Traces in black are for 2-fold dilutions of inhibitor from 62 to 0.98 nM. Curve fitting for two-component models is shown in red.

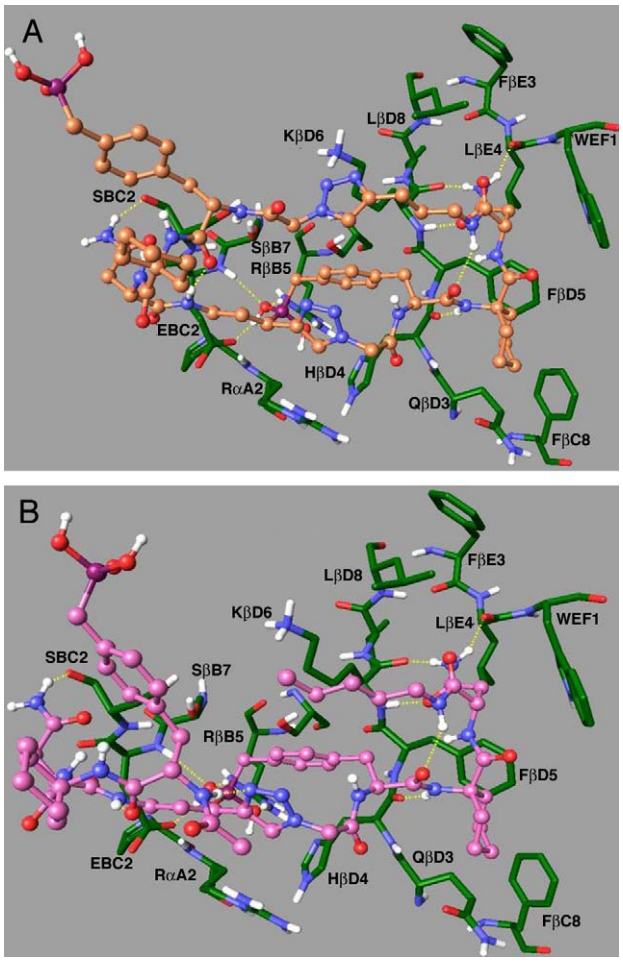


Figure 3. Potential Grb2 SH2 domain-binding interactions of synthetic ligands as determined by molecular modeling. (A) Dimeric macrocycle **13a**; (B) open-chain dimer **18**.

Reported herein is the first application of [3+2] Huisgen cycloaddition click chemistry for the synthesis of SH2 domain-binding peptides. By appending azide/alkyne functionality to the N- and C-termini, respectively, macrocyclization could be effected in monomeric or dimeric fashion depending on substrate concentration. Open-chain dimeric analogues could also be prepared by reacting monomers that contained a single azide/alkyne group each. Although the open-chain (*S*)-Pmp-containing monomer exhibited very low binding affinity, dimeric analogues based on the same motif bound with very

high affinity and displayed complex kinetics. These results support the potential utility of click chemistries for the preparation of novel SH2 domain-binding antagonists.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.08.004.

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16. Compound **12a** ^{1}H NMR (CD_3OD) δ 8.21 (s, 1H), 8.00 (d, J = 8.4 Hz, 1H), 7.76 (s, 1H), 7.68 (d, J = 4.8 Hz, 1H), 7.21 (dd, J = 8.0 and 2.4 Hz, 2H), 7.16 (d, J = 8.0 Hz, 2H), 5.18 (d, J = 16.0 Hz, 1H), 4.99 (d, J = 16.0 Hz, 1H), 4.66 (m, 1H), 4.31(t, J = 6.4 Hz, 1H), 3.33 (m, 1H), 3.05 (d, J = 21.6 Hz, 2H), 3.06–2.94 (m, 2H), 2.73 (t, J = 6.0 Hz, 2H), 2.51 (dd, J = 15.6 and 7.2 Hz, 1H), 2.39 (dd, J = 15.6 and 5.6 Hz, 1H), 2.03–1.90 (m, 3H), 1.70 (m, 2H), 1.58 (m, 1H), 1.48–1.32 (m, 4H), 1.15 (m, 1H), 0.79 (m, 1H). MALDI-MS (+ve) m/z: 647 [MH^+].
17. Compound **13a** ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.70 (d, 1H, J = 7.8 Hz), 8.54 (s, 1H), 7.97 (d, 1H, J = 8.0 Hz), 7.45 (s, 1H), 7.42–7.39 (m, 2H), 7.22–7.16 (m, 4H), 6.96 (s, 1H), 4.95 (d, 1H, J = 16.0 Hz), 4.80 (d, 1H, J = 16.2 Hz), 4.75 (m, 1H), 4.36 (dd, 1H, J = 5.4 Hz and 13.2 Hz), 3.23–3.16 (m, 2H), 3.05 (m, 1H), 2.95 (dd, 1H, J = 6.5 Hz and 21.2 Hz), 2.76–2.66 (m, 2 H), 2.58 (t, 2H, J = 7.5 Hz), 2.43 (dd, 1H, J = 4.7 Hz and 15.6 Hz), 2.02–1.14 (m, 12H). FABMS (-ve) m/z 1291.8 [$(M-H)^-$].
18. Compound **8** ^{1}H NMR (400 MHz, CD_3OD) δ 8.06 (d, 1H J = 15.2 Hz), 7.69 (s, 1H), 7.24–7.14 (m, 9H), 5.00 (dd, 2H, J = 45.6 and 16.0 Hz), 4.63 (m, 2H), 4.49 (dd, 1H, J = 6.4 and 5.2 Hz), 4.41 (t, 1H, J = 6.0 Hz), 3.21–3.02 (m, 5H), 3.05 (d, 4H, J = 21.2 Hz), 2.93 (dd, 1H, J = 14.0 and 9.2 Hz), 2.85–2.63 (m, 6H), 1.84 (s, 3H), 2.00–1.66 (m, 8H), 1.52–1.06 (m, 18H), 0.85 (t, 3H, J = 7.2 Hz). FABMS (-ve) m/z 1254.5 [$(M-H)^-$].
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